" Evaluation of Antiurolithiatic activity of ethanolic extract of Phyllanthus urinaria against ethylene glycol induced urolithiasis in wistar albino rats."

Dissertation submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY

CHENNAI

In partial fulfillment of the requirements for the degree of

MASTER OF PHARMACY in PHARMACOLOGY

BY

Mr. J. ANAND RAJ KUMAR (Reg. No. 261226002)

Under the guidance of

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Associate Professor and Head

Department of Pharmacology

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April-2014

CERTIFICATE

This is to certify that the dissertation entitled **"Evaluation of Antiurolithiatic activity of ethanolic extract of Phyllanthus urinaria against ethylene glycol induced urolithiasis in wistar albino rats."** submitted to The Tamilnadu Dr. M.G.R. Medical university, Chennai, in partial fulfillment for the award of degree of **Master of Pharmacy in Pharmacology** is a bonafide individual research work done by **Mr. J. ANAND RAJ KUMAR** Mohamed Sathak A. J. College of Pharmacy, Chennai, under the guidance and direct supervision of **J.GUNASEKARAN M.Pharm, Associate Professor, Department of Pharmacology** during the academic year 2013-2014.

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(J.GUNASEKARAN, M.Pharm)

Guide and Supervisor

Dedicated to my

Parents, Teachers

&

Friends

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DECLARATION OF THE CANDIDATE

I hereby declare that the thesis titled **"Evaluation of Antiurolithiatic activity of ethanolic extract of Phyllanthus urinaria against ethylene glycol induced urolithiasis in wistar albino rats."** submitted in partial fulfillment for the award of degree Master of Pharmacy to The Tamilnadu Dr. M.G.R. Medical University and carried out at Mohamed Sathak A.J.College of Pharmacy, Chennai, is my original and independent work done under the direct supervision and guidance of **J.GUNASEKARAN M.Pharm, Associate Professor, Department of Pharmacology** during the academic year 2013-2014 and this thesis contains no material which has been accepted for the award of any degree or diploma of other Universities.

Place: Chennai

Date: **[J.ANAND RAJ KUMAR]**

Acknowledgment

ACKNOWLEDGEMENT

I take this opportunity to express my heartfelt thanks to all those, who knowingly or unknowingly contributed to the success of my dissertation work.

I wish to express my gratitude towards "GOD – Almighty", who gave me the strength and courage to fulfill my dream and has showered upon me his choicest blessings.

My heartfelt thanks to my parents and my brother for their love affection and constantly encouraging, guiding when I thought nothing is happening.

I wish to express my deepest gratitude to **Management of Mohamed Sathak trust**, Chennai and **Management of Mohamed Sathak A.J. College of Pharmacy,** in acknowledging all facilities provided to use at the institution enabling us to do work of this magnitude.

I express my sincere thanks to **Dr. R. Sundhararajan,** M.Pharm., Ph.D., Principal, Mohamed Sathak A.J. College of Pharmacy, for his moral encouragement and providing necessary facilities required for my dissertation work.

It is indeed a great pleasure to express my deep sense of gratitude and humble thanks to my guide **J.GUNASEKARAN M.Pharm, Associate Professor &** Head of the Department of Pharmacology,Mohamed Sathak A.J. College Of Pharmacy, Chennai, for his invaluable guidance and constant encouragement that formed the foundation of this project. His discipline, principle, simplicity, the profound knowledge and the subject understanding influenced me a lot. I am proud to say that it has been a most fruitful and enjoyable experience to work under his untiring and dynamic guidance.

I am deeply indebted to the teaching staff especially **Dr. Deepa Sankar,** M. Pharm., Ph.D., Vice principle, Mohamed Sathak A.J. College of Pharmacy, **Mrs. M. Komala,** M. Pharm, (Ph.D)., HOD and **Mrs. N. B. Santha Sheela,** M. Pharm., (Ph.D)., Assistant professor, Department of Pharmaceutics and other teaching staff including **Mr. S. Ramachandran, Mr. Shakti Saravanan,** M. Pharm., who was always a source of knowledge and inspiration to me and also for their prompt assistance and cooperative attitude.

I thank **Mr. A. Mohamad Jamaludeen,** lab assistant, Department of Pharmacology for his timely help.

I wish to express my special thanks to librarians **Dr. M. Amudha, M.A.L.I.Sc., Ph.D.,** and **Mrs. Kumari, M.A.L.I.Sc.,** for helping me in collecting my reference material.

I also wish to express my sincere thanks to **Mr. C. Ramesh,** M. Pharm., Ph.D., Director, SICRA labs, Hyderabad, for his technical support and advices given during the entire course of my project work. With his dynamic approach he boosted my morale, which helped me in completion of this dissertation.

I would like to thank **Mr. Firasat Ali**, M. Pharm., and **Mr. Zuber Ali**, M. Pharm., project officers for their valuable guidance, innovative advices, technical and moral support.

Friends are integral part of life, so I take this opportunity to thank my dearest friends **A.Pruthvidhar, Neelima, Saravanan, Dinesh Kumar, Jaya Kumar** who always pushed my confidence and creativity to the eventual extent of my mind and for their unflinching support and co-operation during my dissertation.

Also I want to thank all teaching and non teaching staff, who directly or indirectly helped me in completing this dissertation work successfully.

Thank you all……..

J. Anand Raj Kumar.

LIST OF ABBREVATIONS

- **AAP:** American Academy of Pediatrics

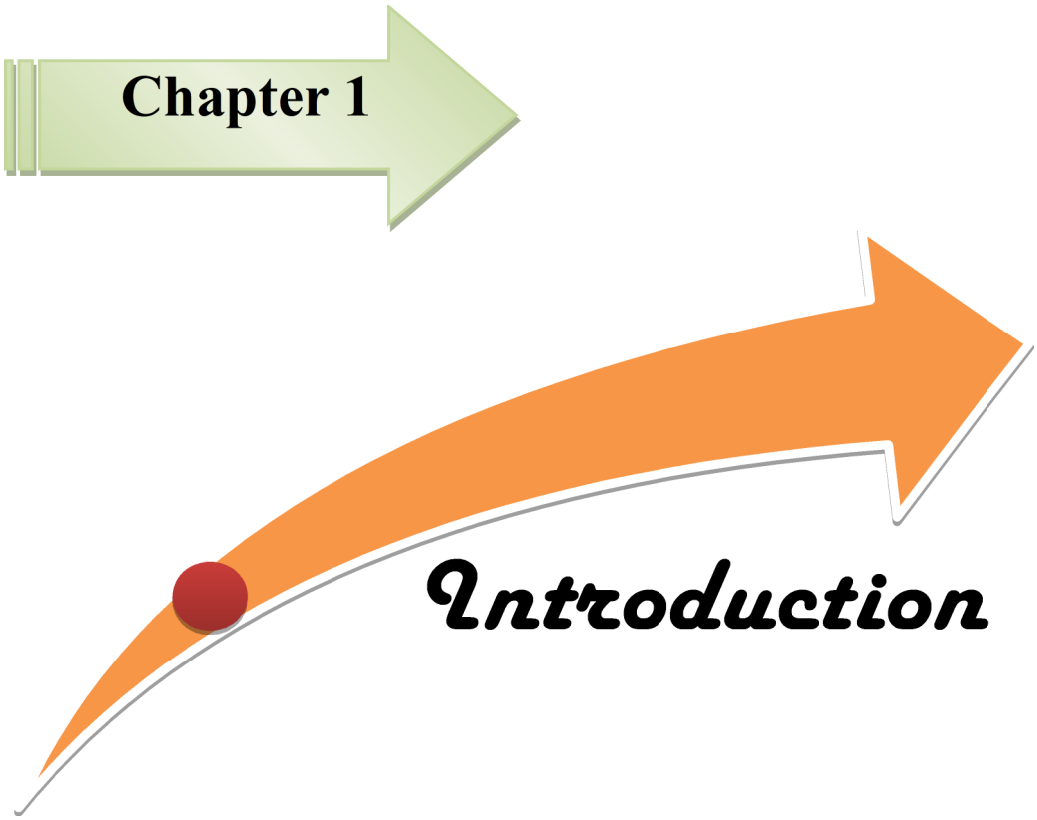
- **AHA:** American Heart Association
- **AMA:** American Medical Association's
- **ATP III :**Adult Treatment Panel III
- **ACAT:** Acyl coenzyme A cholesterol O-acyl transferase
- **ACOA:** Acetyl coenzyme A
- **ANOVA:** Analysis of variance
- **ATP:** Adenosine triphosphate
- **BSCL2:** Berardinelli-Seip congenital lipodystrophy, type 2
- **BMI:** Body mass index
- **CAT:** Catalase
- **CETP:** Cholesteryl ester transfer protein
- **CHD: Coronary heart disease**
- **CHE:** Cholesteryl ester
- **CHOD POD:** Cholesterol oxidase peroxidase
- **CMC:** Carboxy methyl cellulose
- **CPCSEA:** Committee for the purpose of control and supervision of experimental animals
- **CRP:** C-reactive protein
- **DMSO:** Dimethyl sulfoxide
- **FFA:** Free fatty acid
- **GPO:** Glycerol phosphate oxidase
- **GPx:** Glutathione peroxidase
- **GSH:** S-glutathiolation
- **HDL-C:** High density lipoprotein cholesterol
- **HFD:** High fat diet **HL:** Hepatic lipase

- **HMG-COA:** 3-hydroxy 3-methyl glutaryl-CoA reductase
- **HNE:** 4-hydroxy-2-nonenal
- **HOCL:** Hypochlorous acid
- **IDL:** Intermediate density lipoprotein
- **JNC7:** Joint National Committee 7
- **LCAT:** Lecithin cholesterol acyl transferase
- **LDL-C: Low density lipoprotein cholesterol**
- **LLD:** Lipid lowering drugs
- **LPL:** Lipoprotein lipase
- **LRP:** LDL receptor related protein
- **MDA:** Malondialdehyde
- **MI:** Myocardial infraction
- **MVA:** Mevalonate
- **NCEP:** National Cholesterol Education Program
- **NHLBI:**National Heart Blood and Lung Institute
- **NNT** :number needed to treat
- **NNH:** number needed to harm
- **OTC:** over-the-counter
- **OECD:** Organization for economic co-operation and development
- **P.O:** Per oral
- **PPARα:** Paroxisome proliferator activated receptor α
- **SEM:** Standard error mean
- **SOD:** Superoxide dismutase

- **SJM :** Syzygium jambos(L) alston
- **TBA:** Trichloro butyric acid
- **TC:** Total cholesterol
- **TCA:** Trichloro acetic acid
- **TG:** Triglycerides
- **TLC:** Thin layer chromatography

- **TLC:** Therapeutic lifestyle change
- **VLDL:** Very low density lipoprotein cholesterol
    - **WHO**: World health organization

CONTENTS



1. INTRODUCTION

1.1 Definition

Urolithiasis: It is a consequence of complex physical processes. The major factors are super saturation of urine with the offending salt and crystallization. Crystals retained

in kidney can become nucleus for stone formation. This process is known as Urolithiasis or nephrolithiasis.

1.2 Classification of renal stones

Urolithiasis or the renal stones which are formed in the kidneys of various forms based on their chemical composition and their structures over 90 % of stones contain uric acid, urates, oxalate or phosphates, the last two being most commonly found. Based on the predominant chemical composition, Urolithiasis are classified as

1.2.1. Calcium containing stones

a. Calcium oxalate stones (CaOx)

b. Calcium phosphate stones (CaPh)

1.2.2. Uric acid stones

1.2.3. Struvite stones

1.2.4. Cystine stones and miscellaneous stones[1-2]

1.2.1 Calcium containing stones

Calcium stones are the most common type of kidney stones, accounting for about 70% to 75% of stones. The highest incidence of calcium urolithiasis is between ages of 30 and 50 calcium stones either are composed entirely of CaOx or have a small core of CaPh. The presence of a small amount of CaPh has no implications for pathogenesis or management.

1.2.1.a Calcium oxalate stones

CaOx in pure form or in combination with CaPh is the most common component of urinary stones. It generally varies from 4-8 mm in size. The commonest form of oxalate is as calcium oxalate, mainly calcium oxalate monohydrate [Ca $(COO)_2$, H_2O], but some calcium oxalate di hydrate crystals [$Ca(COO)_2$, $2H_2O$] may also be present. Oxalate stones are among the hardest found and so are not easily crushed.

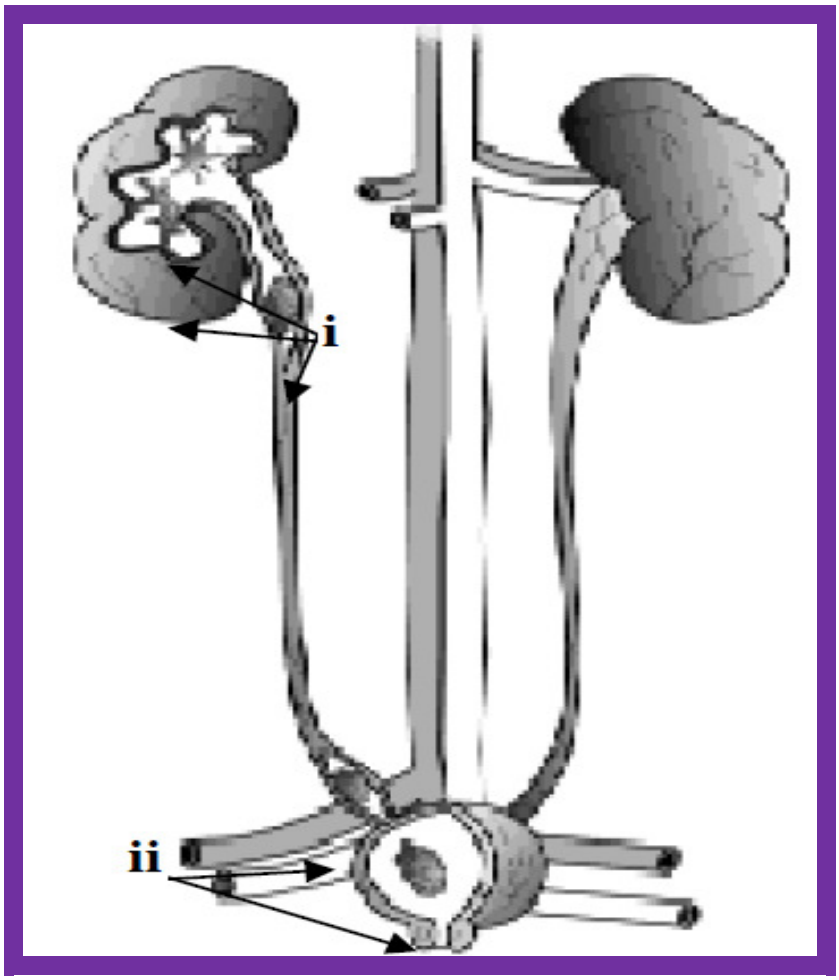


Fig 1: Stones in kidney (i) ureter and (ii) bladder

Idiopathic hypercalciuria: Hypercalciuria in the presence of normal serum calcium is termed *idiopathic hypercalciuria*. The various causes of idiopathic hypercalciuria are as follows:

- **Absorptive hypercalciuria:** The primary defect in absorptive hypercalciuria, inherited as an autosomal dominant trait, is increased passive mucosal absorption of calcium and oxalate in the jejunum.
- **Renal hypercalciuria:** In renal hypercalciuria, the underlying abnormality is a primary renal wasting of calcium. Urinary losses of calcium reduce serum calcium levels and cause a secondary elevation in parathyroid hormone (PTH). This results in 1, 25-dihydroxy-vitamin D production and an increase in intestinal calcium absorption.

Resorptive hypercalciuria: The condition that results in hypercalciuria is increased bone resorption, usually caused by subtle hyperparathyroidism. These patients have parathyroid adenomas but do not have impressive hypercalcemia.

Primary hyperparathyroidism: Primary hyperparathyroidism is caused by a PTH-secreting adenoma of the parathyroid glands. PTH increases serum calcium by the following mechanisms:

1. PTH stimulates osteoclasts to demineralize bone by breaking down the bone crystal apatite. The dissolution of apatite results in the release of calcium and phosphate into the blood stream.

2. PTH causes calcium resorption by the kidneys and decreases renal absorption of phosphates.

3. PTH stimulates production of 1, 25-dihydroxy vitamin D_3 by the kidneys, which in turn increases intestinal resorption of calcium. PTH does not seem to have an effect on intestinal calcium absorption.

Hypercalcemia causes hypercalciuria, which predisposes to urinary calcium stone formation. Decreased urinary citrate has been seen in hyper parathyroid patients.

Hypercalcemia of non parathyroid origin: Common causes of hypercalcemia-causing urinary stone disease, include granulomatous diseases, hyperthyroidism, glucocorticoid-induced hypercalcemia, pheochromocytoma, immobilization and thiazide diuretics. These can be distinguished from hyperparathyroidism by serum parathyroid hormone levels. Serum PTH is elevated in primary hyperparathyroidism, whereas it is generally lower in other hyper calcemias.

Low urinary citrate: Citrate complexes urinary calcium and reduces its ionic concentration. It inhibits spontaneous and heterogeneous nucleation of CaOx crystal. Citrate restores the inhibitory activity of Tomm-Horsfall protein. The most important cause of hypocitraturia is metabolic acidosis, which causes increased proximal tubular reabsorption of citrate. Hypocitraturia is seen in 15% to 63% of patients with urolithiasis.

Hyperoxaluria: Oxalate is the anion most frequently associated with calcium in the precipitation of salts leading to crystal formation, growth, retention and stone

formation. 80 % of urinary oxalate is endogenous in origin and 10% is dietary in origin. Hyperoxaluria results from various factors like diet, genetic predisposure, intestinal diseases and others.

- **Dietary hyperoxaluria:** Normal people excrete 20 – 40 mg (222 - 444 µmol) of oxalate. A reasonable upper limit of excretion is 45 mg (500µmol) daily for men and 40 mg for women. A simple dietary excess of oxalate of 50- 60 mg (556-667µmol) is possible from foods such as spinach, rhubarb, Swiss chard, cocoa, beets, peppers, wheat germ, pecans, peanuts, okra, chocolate and lime peel. This type of hyperoxaluria is frequently observed in nephrolithiasis.
- **Primary hyperoxaluria:** Two genetic disorders lead to hyperoxaluria. Type I primary oxaluria, an autosomal recessive trait is caused by molecular abnormalities that reduce the activity of hepatic peroxisomal enzyme alanine: glyoxylate aminotransferase, thereby increasing the availability of glyoxylate, which is irreversibly converted to oxalic acid. Type II primary hyperoxaluria or L-glyceric aciduria is a much rare variant caused by deficiencies of hepatic enzymes D-glycerate dehydrogenase and glyoxylate reductase, which causes an increase in urinary oxalate (135- 270 mg) and glycerate excretion.

Enteric hyperoxaluria: Occurs in patients with short bowel syndrome or malabsorption. The pathogenesis is exposure of the colonic mucosa to detergents in the form of bile salts and fatty acids which nonselectively increases the permeability to numerous molecules including oxalate. Hyperoxaluria causes oxalate crystal formation, which combines with urinary calcium to form CaOx stones.

Hyperuricosuria: Excessive dietary intake of purine is the most common cause of hyperuricosuria. An abnormality in the renal handling of urate is seen in such patients. Uric acid promotes CaOx crystallization by facilitating the formation of nuclei. Sodium hydrogen urate and uric acid crystals can initiate CaOx crystal formation in seeded solution; however, sodium hydrogen urate crystals are not seen in fresh urine or kidney stones.

1.2.1.b Calcium phosphate stones

Calcium phosphate stones occur only when the chemical pressure for crystallization is high and thus they are usually seen in very active stone disease. Pure Calcium

phosphate stones are almost always associated with the renal tubular acidification defects. When the kidneys lose some of their ability to lower urinary pH, the resulting higher pH increases the divalent and trivalent forms of phosphate, which causes Calcium phosphate supersaturation[3-4].

1.2.2 Uric acid stones

Uric acid calculi account for 5 to 10 % of all stones. The principal cause of uric acid crystallization is the super saturation of urine with respect to un dissociated uric acid. The most important risk factor is acid urine, with the pH persistently below 6.0 and also infections of urinary tract.

1.2.3 Struvite stones

Struvite stones are composed of magnesium, ammonium and phosphate mixed with carbonate. These stones are formed in urine with a pH of greater than 7.2 and ammonia in the urine, produced by urease-producing bacteria. Urease hydrolyzes urea into ammonia and carbon dioxide. The ammonia formed combines with hydrogen to form ammonium. The carbon dioxide formed is hydrated to carbonic acid, which dissociates to bicarbonate ion and a proton. The reaction is illustrated as follows:

$$NH_2CONH_2 + H_2O \leftrightarrow 2\ NH_3 + CO_2$$

$$2\ NH_3 + H_2O \leftrightarrow 2\ NH_4^+ + OH^-$$

$$CO_2 + H_2O \leftrightarrow H^+ + HCO^3 \leftrightarrow H^+ + CO_3^{2-}$$

The responsible bacteria include *Proteus* species, *Klebsiella*, *Serratia*, enterobacteria, *Pseudomonas* and *Staphylococci*. Amongst these, *Proteus mirabilis* is the most common organism associated with struvite calculi. The other mechanism by which bacterial infection can induce stone formation is by increased crystal adherence. Ammonia damages the glycosaminoglycan layer lining of the bladder mucosa, which in turn increases the adherence of struvite crystals in the bladder[5].

1.2.4 Cystine stones

Cystine stones account for less than 5 % of all calculi and occur only in patients with cystinuria. Cystinuria is an autosomal recessive disorder of transmembrane cystine transport manifested in the intestine and in the kidneys[6].

1.3 Causes of renal stone formation

In majority of patients more than one abnormal stone risk factors are involved. It include a mixture of metabolic and environmental factors like gout, renal tubular acidosis, hypocitraturia, hyperoxaluria, hyperparathyroidism, urinary tract infections, cystinuria and hyperuricosuric hypercalciuria are contributory factors for the formation of urinary stones.

1.4 Mechanism of stone formation

1.4.1 Physical concepts

The major factors of urolithiasis are supersaturation and crystallization, inhibitors, complexors, promoters and matrix. The sequence of events leading to stone formation is as follows:

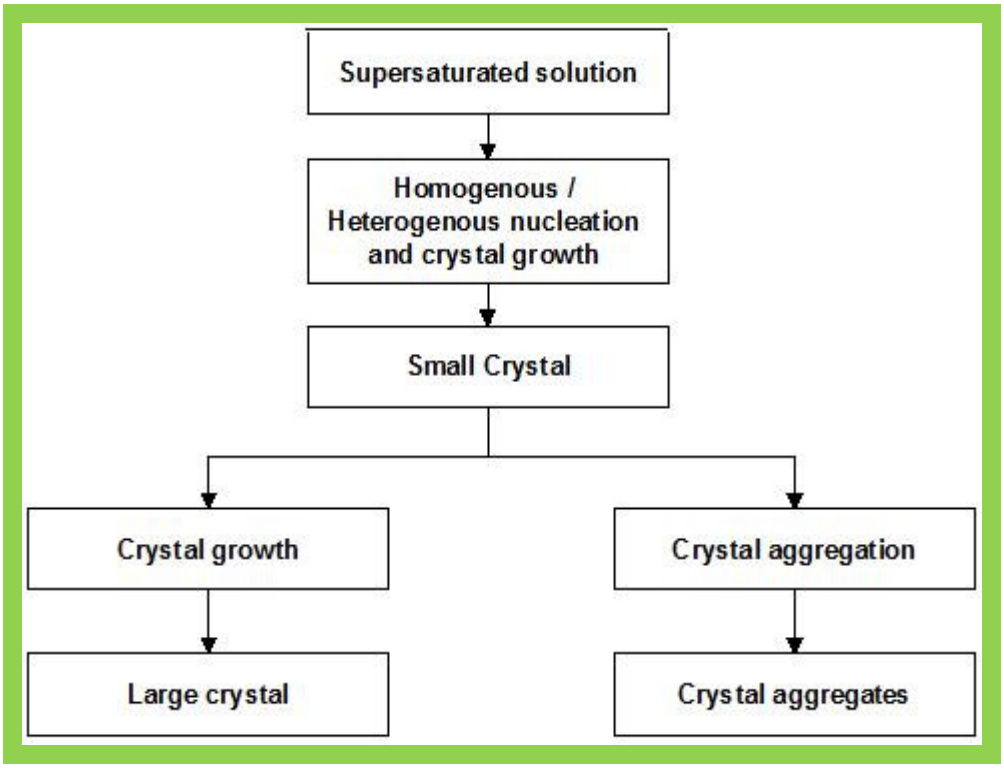


Fig 2: Mechanism of stone formation

Saturation, Supersaturation and Thermodynamic Solubility Product

1.4.2 Pathogenesis of stone formation

Renal stone formation requires that stone forming crystalloids in urine come out of solution. Because crystalloids in solution are in equilibrium with crystalloids in the solid phase, a minimum condition is that urine be supersaturated with relevant crystalloids. This condition is often met: many healthy persons, probably the majority, have concentrations of calcium and oxalate in urine such that their activity product exceeds the solubility threshold (i.e. urine is supersaturated with these crystalloids). But urine has a strong inhibitory action that prevents crystallization and other stone forming processes. Three processes promote stone formation:

- Nucleation,
- Aggregation, and
- Crystal growth.

Nucleation:

Nucleation involves the association of crystalloids in solution (e.g. calcium and oxalate) to form a sub microscopic particle of about 100 atoms. The process requires energy and is facilitated when an external surface can serve as a lattice or anchor, thereby lowering the free energy requirement. Such a surface is provided by microscopic uric acid moieties, which function as promoters of CaOx stone formation.

- **Homogeneous nucleation:** The process by which the earliest crystal nuclei form in pure solutions is called homogeneous nucleation. It occurs in the absence of a surface or lattice.
- **Heterogeneous nucleation:** In this process the nuclei usually form on existing surfaces. Epithelial cells, urinary casts, red blood cells and other crystals can act as nucleating foci in urine.

Aggregation

Aggregation is the process by which nuclei or larger structures adhere to one another. The initial nuclei can grow by the precipitation of additional salt on the lattice framework. It takes between 5 and 7 min for urine to flow from the glomerulus to the collecting duct. The earliest site of stone formation in human is the papillary duct or the collecting tubule, where the diameter is 50 to 200 μm .Once nuclei are formed they bounce apart from each other, float freely and become kinetically active. If they

remain independent and float freely, they are washed away by urine flow. However, under certain circumstances, these nuclei come in close contact and due to chemical or electrical forces can bind to each other, a process called *crystal aggregation*.

Crystal growth

A third process is crystal growth, in which crystalloids come out of solution to associate with the solid phase of growing crystal in a geometrically precise arrangement. The combination of crystal aggregation and crystal growth can explain the genesis of urinary calculi. Another process that may lead to CaOx stone formation is *crystal retention*. In most instances, crystal aggregates are too fragile to occlude the collecting duct long enough to give rise to a stone[7-8].

1.4.3 Promoters, inhibitors and complexing agents:

Each process of stone formation has specific promoters and inhibitors. Glycosaminoglycans promote crystal nucleation but inhibit crystal aggregation. Tamm- Horsfall protein, a product of the thick ascending limb of Henle, may act as a promoter or inhibitor of crystal formation depending on its state of aggregation.

Nucleation of CaOx is inhibited by magnesium and citrate, which forms soluble complexes with calcium and so reduces its effective concentration. a highly negatively charged molecule rich in aspartate and γ-carboxyglutamic acid residues, is potent inhibitor of CaOx crystallization in simple solutions. Aggregation is inhibited by uropontin is an aspartic acid-rich protein that shares the N-terminal amino acid sequences with human osteopontin. It is an important inhibitor of CaOx crystal growth. Citrate is a potent complexor of calcium and reduces the ionic calcium in the urine with consequent reduction in the supersaturation of calcium salt. Citrate exerts maximum effect at a pH of 6.5. Magnesium, a divalent cation, complexes oxalate in the CaOx system[7].

1.5 Signs and symptoms

Kidney stones on accumulation in the urinary tract exhibit specific symptoms and the most specific one is severe pain in the. The common symptoms include, back pain on one or both sides, Progressive severe colicky (spasm-like) may radiate or move to lower in flank, pelvis, groin, genitals, Nausea, vomiting, Urinary frequency/urgency,

increased (persistent urge to urinate), Blood in the urine , Abdominal pain, Testicle pain , Fever, Chills and Abnormal urine color[8]

1.6 Diagnosis (Investigations)

A. Urine analysis: Urine analysis in most patients with ureteral calculi reveals the presence of microscopic or gross hematuria. A urine analysis should be done promptly after collection. The pH is best measured in a morning urine specimen after an overnight fast. If the pH of urine is higher than 7.6, urea splitting organisms must be present, for the kidneys cannot produce urine in this range of alkalinity. Such a finding strongly suggests that the stones are composed of magnesium ammonium phosphate. Fixation of the pH at 6 to 6.5 is compatible with renal tubular acidosis. Consistently low pH is a common cause of the formation of uric acid calculi.

B. Blood investigations

- **Complete blood count:** The white blood count may be increased as a result of complicating infection. If renal function is not adequate, anaemia may be found.
- **Renal function tests:** Blood urea nitrogen and serum creatinine will give an indication of the functional status of the kidney.
- **Serum blood chemistry:** This investigation is usually done in recurrent stone formers. Fasting serum calcium and phosphates should be determined on 3 occasions. Serum proteins should also be estimated, since almost half of the calcium is normally unionized and bound to proteins. Hypercalcaemia is most commonly seen in association with osteolytic or disseminated malignant disease, especially cancers of the breast and lung, multiply myeloma, leukemia and sarcoidosis, but serum phosphate is usually normal. Serum alkaline phosphatase is increased in hyperparathyroidism only if bone disease is present. Elevated serum uric acid is found in 50% of uric acid stone formers.

C. Plain X-ray: A plain film of the abdomen may show a calcific body in the region of the kidney or in the ureter. This constitutes merely presumptive grounds for the diagnosis. Ureteric calculi often looks like root of a tooth, sometime it may be confused with pelvic phleboliths which are usually round.

D. Excretory urography: Excretory urograms are useful in the diagnosis of ureteric calculus. The ureterogram places the calcification in the ureter and also demonstrates dilatation.

E. Ultrasonography: Ultrasonography is a noninvasive method of demonstrating both ureteral calculi and the consequent hydronephrosis. Color dropper ultrasound examination may demonstrate the increased resistive index in the obstructed kidney and assymetry or absence of ureteral jets in urinary bladder.ons of the ureter above the stone.

F. MRI: The study has potential usefulness when patients have renal impairment or allergy to intravenous contrast agents and when X-rays are contraindicated. In contrast to CT, not only MRI is unable to visualize most stones but invitro studies have determined that this modality is not useful for characterizing the composition of stones.

G. Radioisotope methods: Radioisotope renography and scanning have contributed to the diagnosis of obstruction and location of calculus in a number of patients, particularly those who are otherwise sensitive to contrast media. This technique is helpful not only to the patients sensitive to the intravenous contrast and knowing the location of the calculus but also the degree of urinary obstruction is clearly defined.

H. Instrumental examination: Cystoscope and ureteroscope is seldom needed for the diagnosis of ureteral calculus.

I. Analysis of stone: Chemical analysis of renal calculi has been all but abandoned. Significant error may occur because qualitative and semiquantitative chemical methods are not accurate. Studies have shown that X-ray diffraction; Infrared spectroscopy procedures are accurate in detecting components of urinary calculi[9].

1.7 How are kidney stones treated?

Fortunately, surgery is not usually necessary. Most kidney stones can pass through the urinary system with plenty of water (2 to 3 quarts a day) to help move the stone along. Often, patient can stay home during this process, drinking fluids and taking pain medication as needed.

1.7.1 The first step: Prevention

If a patient has had more than one kidney stone, he is likely to form another; so prevention is very important. To prevent stones from forming, doctor must determine their cause and he or she will order laboratory tests, including urine and blood tests. Doctor will also ask about patient's medical history, occupation and eating habits. The passed or removed stone has to be analyzed in the laboratory, because its composition helps in planning treatment.

1.7.2 Lifestyle changes

A simple and most important lifestyle change to prevent stones is to drink more liquids; water is the best. If patient tends to form stones, he or she should try to drink enough liquids throughout the day to produce at least 2 quarts of urine in every 24 h period. People who form calcium stones used to be told to avoid dairy products and other foods with high calcium content. However, recent studies have shown that foods high in calcium, including dairy foods, help prevent calcium stones. Taking calcium in pill form, however, may increase the risk of developing stones.

1.7.3 Medical therapy

A 24-hour urine collection with measurement of the important analytes is usually reserved for use in patients with recurrent stone formation. In these patients, the major urinary risk factors include hypercalciuria, hyperoxaluria, hypocitraturia and hyperuricosuria. Effective preventive and treatment measures include thiazides therapy to lower the urinary calcium level, citrate supplementation to increase the urinary citrate level and, sometimes, allopurinol therapy to lower uric acid excretion. Uric acid stones are most often treated with citrate supplementation.

1.7.3 Surgical treatment

Surgery should be reserved as an option for cases where other approaches have failed or should not be tried. Surgery may be needed to remove a kidney stone, if it

- Does not pass after a reasonable period of time and causes constant pain
- Is too large to pass on its own or is caught in a difficult place
- Blocks the flow of urine
- Causes ongoing urinary tract infection
- Damages kidney tissue or causes constant bleeding

Stone episode (resolved)

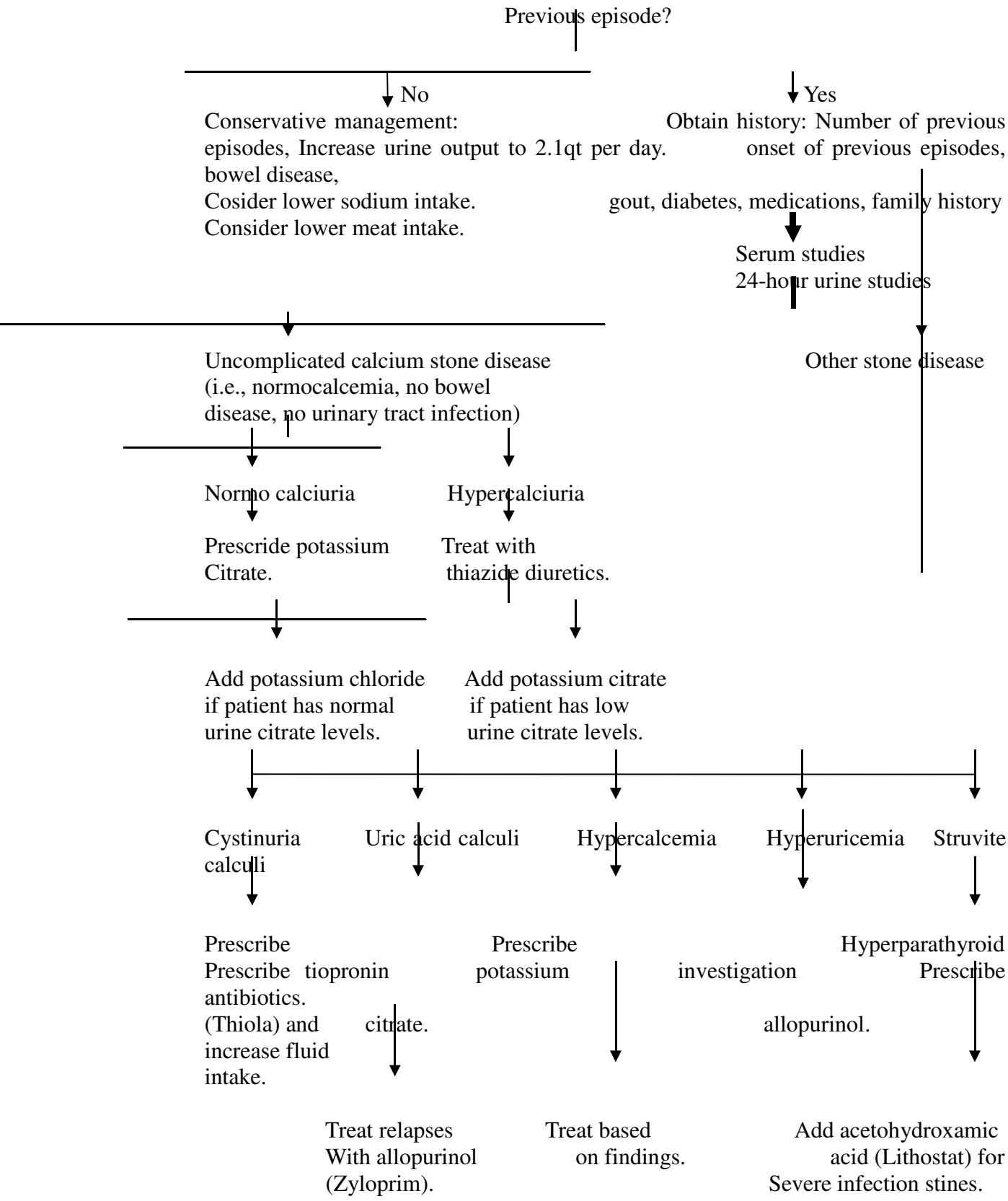


Fig 3: Algorithm for the medical management of recurrent urinary calculi

Until recently, surgery to remove a stone was very painful and required a recovery time of 4 to 6 weeks. Today, treatment for these stones is greatly improved and many options do not require major surgery.

A. Extracorporeal shockwave lithotripsy

Extracorporeal shockwave lithotripsy (ESWL) is the most frequently used procedure for the treatment of kidney stones. In ESWL, shock waves that are created outside the body travel through the skin and body tissues until they hit the denser stones.

The stones break down into sand-like particles and are easily passed through the urinary tract in the urine[10-11].

1.8 Natural products used in the treatments of urolithiasis.

Ammannia baccifera[12], Blackcurrant (*Ribes nigrum*)[13], *Coleus aromaticus* [14], *Costus spiralis* Roscoe[15], Cranberry *(Vaccinium macrocarpon*)[16], *Cyclea peltata*[17], *Herniaria hirsute*[18-19], Lupeol and Betulin[20-21], *Moringa* oleifera[22], *Musa paradisiaca*[23-24], *Phyllanthus niruri*[25-26], *Raphanus sativus*[27], *Salix taxifolia*[28], *Tribulus terrestris*[29-31], Varuna (*Crataeva nurvala)*[32-33], *Crataeva adansonii*[34], *Aerva lanata*[35-36], *Bergenia ligulata* rhizome[37], *Nigella Sativa*[38], *Eleusine Corac*ana[39], *Hibiscus sabdariffa*[40], *Quercus salicina* Blume/*Quercus stenophylla* Makino[41], *Ammi visnaga*[42], *Cynodon dactylon*[43], Orthosiphon stamineus[44], *Rotula aquatica, Commiphora wightii and Boerhaavia diffusa*[45], *Herniaria hirsuta* and *Agropyron repens*[46]*Tephrosia purpurea*[47].

1.9 Ethylene glycol induced urolithiasis:

Ethylene glycol is present in many common substances such as antifreeze, de-icing substances, detergents, lacquers, and polishes. Ethylene glycol itself is not toxic; rather the metabolites, glycolic acid and oxalic acid exert their toxic effects. Three stages of toxicity from ethylene glycol are classically identified: 1) CNS stage, 30 min to 12 hours after ingestion with features of altered mental status, ataxia and slurred speech; 2) cardiopulmonary stage, 12-24 hours after ingestion with hypertension, tachycardia, congestive heart failure, and adult respiratory distress syndrome; 3) renal stage, 24-72 hours after ingestion with flank pain, calcium-oxalate crystalluria, and oliguria[48] Of interesting note, two forms of oxalate crystals can be present in the urine in ethylene glycol poisoning, one more specific than the other. A dumbbell shaped monohydrate crystal is most common, however the dihydrate form is most specific, as

the monohydrate form can be present in those who consume large quantities of vitamin C as well as diets high in urate. The dihydrate form also requires a higher concentration of oxalate to be present, and is thus more indicative of ethylene glycol poisoning[49].

Toxicity results from the depressant effects of ethylene glycol on the central nervous system (CNS).Metabolic acidosis and renal failure are caused by the conversion of ethylene glycol to noxious metabolites. Oxidative reactions convert ethylene glycol to glycoaldehyde, and then to glycolic acid, which is the major cause of metabolic acidosis.[50-51] Both of these steps promote the production of lactate from pyruvate.[52] The conversion of glycolic acid to glyoxylic acid proceeds slowly, further increasing the serum concentration of glycolic acid.4 Glyoxylic acid is eventually converted to oxalic acid and glycine. Oxalic acid does not contribute to the metabolic acidosis, but it is deposited as calcium oxalate crystals in many tissues. Ethylene glycol is rapidly absorbed by the stomach and small intestine, and is quickly redistributed throughout the body. Metabolites of ethylene glycol remain in the body for several days,with calcium oxalate present in tissues for much longer. The clinical syndrome of ethylene glycol intoxication has traditionally been divided into three stages: progressive involvement of the CNS, the cardiopulmonary systems, and the kidneys. However, presentation is highly variable and dependent on the amount ingested, the combined ingestion with ethanol, and the timing of medical intervention.[53]

1.9.1 Clinical Manifestations

Ethylene glycol produces CNS depression similar to that of ethanol. Symptoms of ethyl-ene glycol toxicity include confusion, ataxia, hallucinations, slurred speech, and coma. Symptoms are most severe six to 12 hours after ingestion, when the acidic metabolites of ethylene glycol are at their maximal concentration The presentation may be similar to ethanol intoxication, if the patient presents early or has consumed small amounts of ethylene glycol. However, an ethanol odor will be absent, and serum or respiratory ethanol levels will be too low to account for the degree of CNS depression. The absence of a strong odor of alcohol in a patient who appears intoxicated should raise the suspicion of ethylene glycol ingestion. Following a period of CNS depression, metabolic acidosis and cardiopulmonary symptoms become prominent, although co ingestion of ethanol will delay the metabolic acidosis. The patient may experience nausea, vomiting, hyperventilation, and hypocalcemia with

muscle tetany and seizures. Hypertension, tachycardia, and cardiac failure may ensue. Pneumonitis, pulmonary edema, and adult respiratory distress syndrome have also been reported.[54] Renal involvement may become apparent within 24 to 72 hours after ingestion. Urinary crystal formation requires a sufficient amount of time for ethylene glycol to be metabolized into oxalate. Calcium oxalate formation depletes serum calcium levels and deposits in intestinal mucosa, liver, brain, heart, lung, and kidney. The excretion of calcium oxalate crystals in the urine is usually, but not always, present. Oliguric or anuric renal failure is the result in the most severe cases and, although permanent renal failure is rare, recovery of renal function may take up to two months. If untreated, severe ethylene glycol toxicity is usually fatal within 24 to 36 hours.[55]

1.9.2 Treatment

Current practice guidelines for initiating treatment of ethylene glycol poisoning have been published by the American Academy of Clinical Toxicology (AACT) and are listed in table.

TABLE 3
AACT Criteria for Treatment of Ethylene Glycol Poisoning with an Antidote

Documented plasma ethylene glycol concentration greater than 20 mg per dL (3 mmol per L)
or
Documented recent (hours) history of ingesting toxic amounts of ethylene glycol and osmolal gap greater than 10 mOsm per kg of water
or
History or strong clinical suspicion of ethylene glycol poisoning and at least two of the following criteria:
Arterial pH less than 7.3
Serum bicarbonate level less than 20 mEq per L (20 mmol per L)
Osmolal gap greater than 10 mOsm per kg of water*
Urinary oxalate crystals present

AACT = American Academy of Clinical Toxicology.

**—Laboratory analysis by freezing point depression method only.*

Adapted with permission from Barceloux DG, Krenzelok EP, Olson K, Watson W. American Academy of Clinical Toxicology practice guidelines on the treatment of ethylene glycol poisoning. Ad Hoc Committee. J Toxicol Clin Toxicol 1999;37:538.

Ethylene glycol is rapidly absorbed from the stomach, making treatment with gastric lavage and syrup of ipecac ineffective. Likewise, it requires large amounts of activated charcoal to bind relatively small amounts of ethylene glycol, and the therapeutic window for this action is less than an hour. Traditional treatment of ethylene glycol poisoning consists of sodium bicarbonate, ethanol, and hemo dialysis. Fomepizole is a new agent with a specific indication by the U.S. Food and Drug Administration for the treatment of ethylene glycol poisoning.[56] Ethanol and fomepizole are thought to act as inhibitors of alcohol dehydrogenase and therefore prevent the formation of acidic ethylene glycol metabolites, but only fomepizole has demonstrated this ability. If patients are diagnosed and treated with these products early in the course of poisoning, hemo dialysis may be avoided. Once severe acidosis and renal failure have occurred, however, hemodialysis is necessary. Fomepizole treatment should be initiated immediately when ethylene glycol poisoning is suspected. Within three hours of initiating therapy with fomepizole, inhibition of metabolite production and resolution of acidosis occurs, and the anion gap is normalized within four hours. If fomepizole therapy is begun before a rise in the serum creatinine concentration, damage to the kidney can be avoided. When compared with ethanol, the advantages of fomepizole include a slower rate of excretion by the kidneys, lack of CNS depression or hypoglycemia, and easier maintenance of effective plasma levels.

Ethanol may be administered orally or intravenously. The recommended therapeutic blood ethanol level is 100 to 150 mg per dL (22 to 33 mmol per L). The AACT provides specific dosage recommendations for ethanol in patients receiving standard treatment and patients on hemodialysis. While oral ethanol can be simply administered, it requires a conscious patient who is willing to drink the ethanol or tolerate the placement of a nasogastric tube. Advantages of intravenous infusion include greater absorption and no gastrointestinal upset. Disadvantages of treatment with ethanol include variable metabolism of ethanol; inebriation and CNS depression; frequent monitoring of serum concentrations (every one to two hours); difficulty maintaining effective serum concentrations; and the need to administer ethanol in an intensive care unit. Administration of intravenous sodium bicarbonate will correct the metabolic acidosis, increase the elimination of renal glycolic acid, and inhibit the precipitation of calcium oxalate crystals, although the latter benefit has not been proved in clinical trials. Fifty to 100 mEq per L of intravenous fluid is usually sufficient, with a goal of maintaining a urine pH greater than 7.0.3 If the diagnosis of

ethylene glycol poisoning is not made, and the acidosis is treated only with bicarbonate, organic acids will continue to be produced.10 Treatment with sodium bicarbonate may worsen hypocalcemia initially because of the protein binding of calcium. Hemodialysis is effective in removing ethylene glycol and glycolic acid, and correcting the metabolic acidosis.[57] A serum ethylene glycol concentration greater than 50 mg per dL (8 mmol per L) by itself is no longer considered a criterion for hemodialysis. In the absence of renal dysfunction and significant metabolic acidosis, the use of fomepizole should eliminate the need for hemodialysis in patients with high serum ethylene glycol concentrations; in these patients, frequent monitoring of acid-base balance is necessary. When ethanol or fomepizole is administered and renal failure is present, dialysis is the only method for removal of ethylene glycol. If metabolic acidosis persists, too little ethanol or fomepizole is being administered. Traditionally, hemodialysis is continued until ethylene glycol and glycolic acid levels cannot be detected in the blood, and there are no acid base disturbances. Prolonged dialysis may not be necessary in patients treated with fomepizole or ethanol; the end point for dialysis in these patients is correction of the anion and osmolar gaps. Serum osmolality levels and electrolyte levels should be monitored closely every two to four hours for 12 to 24 hours following the discontinuation of dialysis because redistribution of ethylene glycol may result in an elevated serum concentration. Pyridoxine (vitamin B6) and thiamine (vitamin B1) in dosages of 100 mg daily are believed to promote the conversion of intermediate byproducts into nontoxic metabolites, but clinical data supporting their effectiveness do not exist. Therapy with 100 mg of intravenous thiamine would be appropriate if ethanol withdrawal is suspected. Parenteral calcium, given as gluconate or chloride salts, may be necessary for treatment of tetany and seizures caused by hypocalcemia. [58]

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE

Lin SY *et al* reported Antioxidant, anti-semicarbazide-sensitive amine oxidase, and anti-hypertensive activities of geraniin isolated from Phyllanthus urinaria, Geraniin also showed dose-dependent inhibitory activities against semicarbazide-sensitive amine oxidase (SSAO, IC50 were 6.58 microM) and against angiotensin converting enzyme (ACE, IC50 were 13.22microM). For kinetic property determinations, geraniin showed competitive inhibitions against SSAO (the apparent inhibition constant, Ki, was 0.70microM) and mixed noncompetitive inhibitions against ACE. The geraniin showed antihypertensive activity in lowering SBP and DBP and showed a significant difference from the blank (distilled water) at 2, 4, 6, 8, and 24 h. Healthy food products could use geraniin for antioxidant protection and therapeutic effects in the future.

Lee et al reported Hepatoprotective effect of Phyllanthus in Taiwan on acute liver damage induced by carbon tetrachlorideThe effect of oral administration of Phyllanthus methanolic extracts (PME) (P. urinaria L.s. urinaria is hepatoprotective and antioxidant agents.

Chularojmontri L et.al reported Antioxidative and cardioprotective effects of Phyllanthus urinaria L. on doxorubicin-induced cardiotoxicity.

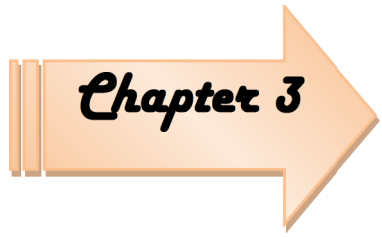
Shen ZQ ,et al investigated the effects of PUW (a fraction containing 60% corilagin from a Chinese herbal plant Phyllanthus urinaria) on thrombosis and coagulation system.They showed that PUW administered intravenously significantly decreased the mouse mortality, prolonged the occlusion time of rat carotid arteries, and reduced the wet and dry thrombus weight of the inferior vena cava, respectively. PUW markedly inhibited the binding of activated platelets to neutrophils, obtaining 39.7 mg/L of the medium inhibitory concentration. Intravenously administered PUW significantly shortened ELT, prolonged KPTT while had no influence on PT; PUW increased BT in rat tail tips but the BT caused by PUW was much shorter than that by aspirin or urokinase.

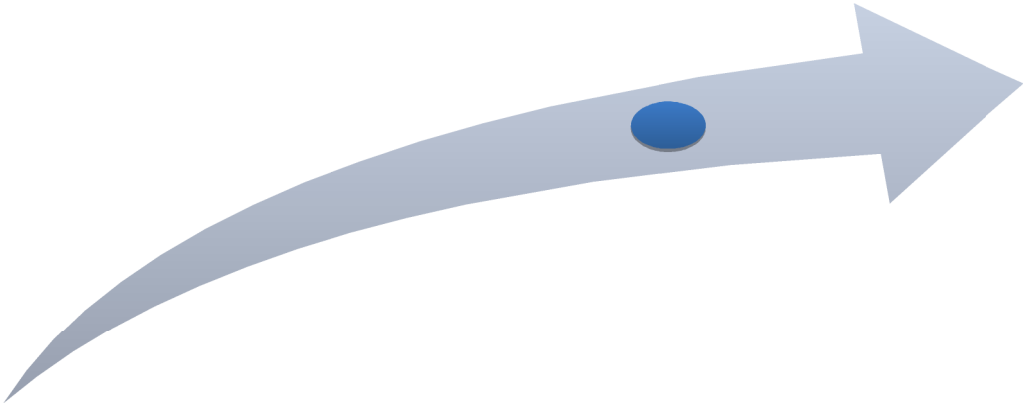
Zhang LZ et al reported Isolation and identification of a novel ellagitannin from Phyllanthus urinaria L. Phyllanthusiin G is a new compound was isolated. A novel ellagitannin named phyllanthusiin G was isolated, its structure was established as 1-O-galloyl-2-phyllanthoyl-3,6-(R)-HHDP-beta-D-glucose.

Xu M, et al reported Phenolic antioxidants from the whole plant of Phyllanthus urinaria The 1,1-diphenyl-2-picrydydrazyl (DPPH) assay on the extract of Phyllanthus urinaria L. (Euphorbiaceae) displayed considerable radical-scavenging activity (SC50 = 14.3 microg/ml). Further bioassay-guided purification of the extract led to the isolation of a series of 15 phenolic compounds, including the ellagitannins 1-7, the flavonoids 8-10, and the simple hydroxylated (or glycosylated) aromatic acids 11-15. Their structures were identified by spectroscopic analyses and comparison with authentic samples or literature data. The structure of repandinin B (1) was for the first time fully assigned by 1D- and 2D-NMR experiments. The phenolic compounds 1, 3, 4, 6, 9, 11, and 15 have not been isolated before from the title plant.

Zhong Y et al reported Chemical constituents of Phyllanthus urinaria L. and its antiviral activity against hepatitis B virus. Studies on the chemical constituents of Phyllanthus urinaria and its antiviral activity against hepatitis B virus were completed. Eleven compounds have been isolated. Two of them are new compounds methyl ester dehydrochebulic acid and methyl brevifolin carboxylate. Antiviral experiments on HBsAg in vitro and liver damage caused by CCl4 have shown that. Phyllanthus urinaria possesses antiviral activities against HBV.

Paulino N, et al reported The relaxant effect of extract of Phyllanthus urinaria in the guinea-pig isolated trachea. Evidence for involvement of ATP-sensitive potassium channels. indicate that the ATP-activated potassium channels sensitive to glibenclamide, but not the small conductance calcium-activated potassium channels sensitive to apamin, largely contribute to the relaxation effect of the hydroalcoholic extract of P. urinaria in GPT. In addition, both beta 2 and VIP-mediated responses seem to account, at least in part, for the relaxation effect of the hydroalcoholic extract, as its relaxant response was partially attenuated by both propranolol and VIP receptor antagonist.





AIMS & OBJECTIVES

3.1 Aims:

Urolithiasis is defined as the presence of one or more calculi in any location within the urinary tract. Urolithiasis is the third most common disorder of the urinary tract, the others being frequently occurring urinary tract infections and benign prostatic hyperplasia. The worldwide incidence of urolithiasis is quite high and in spite of tremendous advances in the field of medicine, there is no truly satisfactory drug for the treatment of renal calculi. Most patients still have to undergo surgery to be rid of this painful disease. Hyperoxaluria is the main initiating factor for urolithiasis. *Phyllanthus urinaria* is said to be useful in the treating urinary calculi. Hence the present study an effort has been made to establish the scientific validity for the anti-urolithiatic activity.

3.2 OBJECTIVES OF THE STUDY:

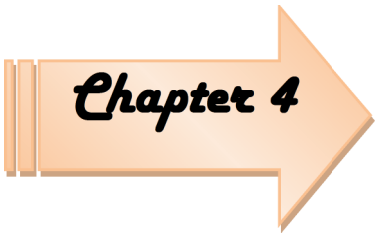
1. To prepare ethanolic extract of *Phyllanthus urinaria*

2. To study the phytochemical screening of ethanolic extract *Phyllanthus urinaria*

3. To study the acute oral toxicity ethanolic extraction of *Phyllanthus urinaria*

4. To study the antiurolithiatic activity of ethanolic extract of *Phyllanthus urinaria in* rats

5. To carry out histopathological studies.



PLANT PROFILE

4. PLANT PROFILE

4.1 Plant profile of Phyllanthus urinaria:

Phyllanthus urinaria is an annual plant with the main stem erect, un branched or sparsely branched and seldom more than a foot tall. The side branches with their two rows of alternate leave resemble a compound leaf. The leaves themselves are finely hairy, nearly sessile (stalkless), oblong to narrowly obovate.

Phyllanthus urinaria

Rank	Scientific Name and Common Name
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Euphorbiales
Family	Euphorbiaceae
Genus	*Phyllanthus* L.
Species	*Phyllanthus urinaria* L.

4.2 Common name :

Chanca piedra, shatterstone, meniran, stone breaker, quebra pedra, zhen chu cao, ye xia zhu, chamber bitter, kilanelli, leafflower, komikansou

4.3 Suriname's traditional medicine

It is used against colic, and as an effective remedy to eliminate gall - and kidney stones, urinary tract infection (UTI), bladder inflammation and for other kidney and liver problems in general such as acute - and chronic Hepatitis B.

4.4Overview;

Shatterstone is a small tropical annual herb growing up to 2 feet tall. Along the erect, red stem are equally set small green, oblong feathered leaves. It has greenish white flowers. A very small wart-like fruit, greenish-red, is underneath every pair of the feathered leaves. When the plant is picked the feathery leaves fold in, completely closing themselves. The plant is used for several conditions such as blennorrhagia (gonorrhea), diabetes, dysentery, flu, tumors, jaundice (the yellow color of the skin and whites of the eyes caused by excess bilirubin in the blood), vaginitis (swelling, itching, burning or infection in the vagina), against headache, fever, conjuntivitis (pinkeye or bloodshot eyes), menstrual disorders and dyspepsia (pain or an uncomfortable feeling in the upper middle part of the stomach).

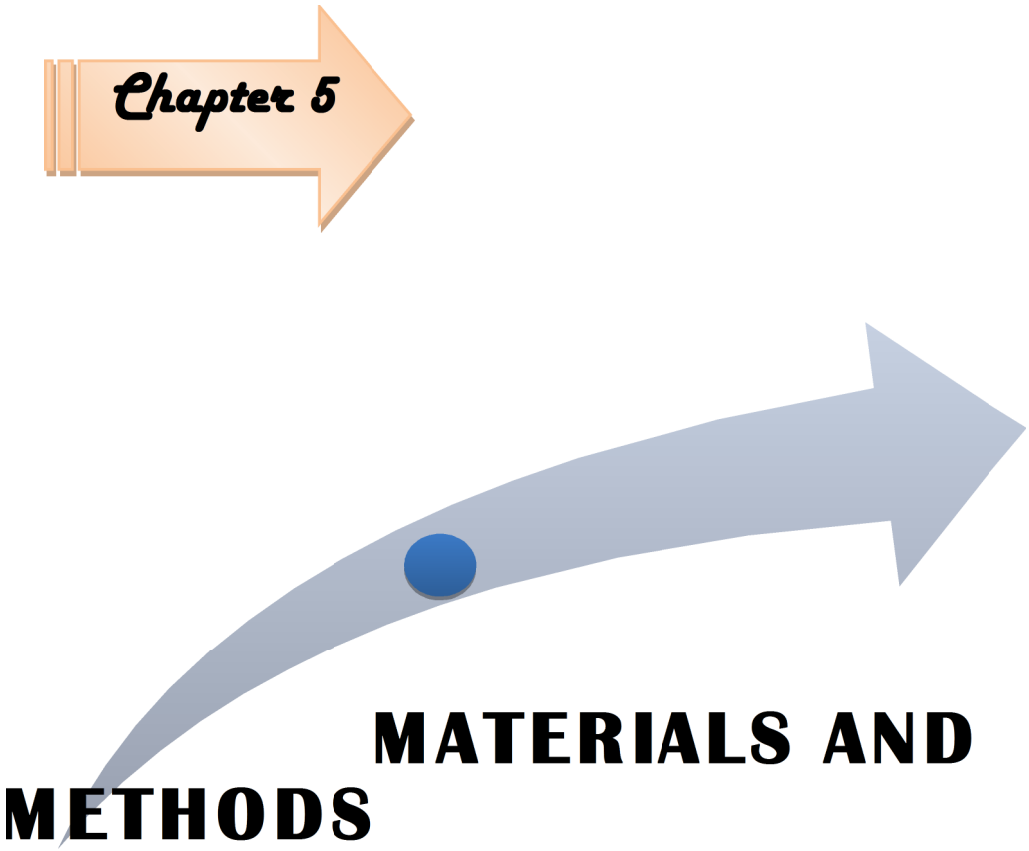
in treating liver- and kidney ailments; used extensively for detoxification

4.5 Pharmacology

The primary action of shatterstone is on the liver; it acts by the inhibition of DNA polymerase on the hepatitis B virus. The enzyme DNA polymerase is needed for the virus to reproduce.Several studies suggest that Phyllanthus urinaria works better than the related species p. amarus, p. debilis and p.niruri in the treatment of hepatitis B. An equally important action is the use against kidney stones (renal calculi), urinary tract- and bladder infections. In preliminary research in animals, extracts of Phyllanthus plants have shown promising results in pain relief. The mechanism seems to be that this is reached by decreasing inflammation.

Phyllanthus urinaria (P. urinaria), one of the herbal plants belonging to the genus Phyllanthus (Euphorbiaceae), is widely distributed in China, Southern India and Southern America. It has long been used in folk medicine for the treatment of several diseases such as hepatitis B, neprolithiasis and in painful disorders[59-61] 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4- methylene dioxy lignan isolated from the ethyl acetate extract of P. urinaria was shown to exhibit anticancer activity by inducing apoptosis through the inhibition of telomerase activity and Bcl-2 expres- sion.[62] previous studies also demonstrated that the water extract prepared from P. urinaria has an anti- cancer effect on Lewis lung carcinoma cells through a similar pathway.[63] In addition, we

demonstrated that the anti-tumor and anti-angiogenic effects of P. urinaria in mice bearing Lewis lung carcinoma were due to interference with migration of vascular endothelial cells but not viability.[64] Therefore, P. urinaria became our study target and we prepared the drug with a standardized protocol under regulation and used it for further investigation.



5. MATERIALS AND METHODS

Materials and methods

❖ **Materials-**

Plant material - Plant material: The plant of *Phyllanthus urinaria* plants were collected from the certified ayurvedic wholesaler. The plant was identified and authenticated by Asst. Prof. Dr. K. Madhava chatty, MSc, Med, Department of Botany, S.V. University, Tirupati

Drugs and Chemicals

Cystone (Himalaya Pharmaceutical, Banglore), Ethylene glycol (SRL Mumbai), Tween 80 (Merck Pvt Ltd, B, Mumbai)**,** Anaesthetic ether (SD Finechem Ltd., Mumbai), Chloroform (SD Finechem Ltd .Mumbai). Formaline (SD Finechem Ltd., Mumbai) and all other chemicals and reagents were of analytical grade.

Diagnostic kits:

Diagnostic kits used for estimation of Creatinine, Urea, Uric acid, Calcium, Phosphorus, Calcium oxalate were procured from **Robonik Diagnostic Ltd India.**

Instruments:

Autoanalyzer (Robonik), Refrigerator centrifuge (MPW-350R),UV-Spectro-phot ometer (UV-1601, Shimadzu Corporation, Kyoto, Japan), Mini Lyotrap (LTE Scientific Ltd.), Research centrifuge (Remi industries, Mumbai) and homogenizer (Remi Motors, Mumbai). Dhona balance (M/S Dhona instruments Pvt. Ltd., Kolkata, India).

Experimental Animals:

Wistar albino male (180–220 g) was obtained from the central animal house of Sigma Institute of Clinical Research and administration Pvt Ltd Hyderabad. The animals

were housed at room temperature (22-28 ºC) for 12 hr dark and 12 hr light cycle and given standard laboratory feed and water *ad-libitum*. The study was approved and conducted as per the norms of the Institutional Animal Ethics Committee (180/SICRA/IAEC).

Acute toxicity study

Procedure: Acute toxicity studies were performed according to OECD-423guidelines category IV substance (acute toxic class method). Swiss albino mice (n=3) of either sex selected by random sampling technique were employed in this study. The animals were fasted for 4 hrs with free access to water only. The plant extracts of *Phyllanthus urinaria* were administered orally with maximum dose of 2000 mg/kg body weight. The mortality was observed for three days. If mortality was observed in 2/3 or 3/3 of animals, then the dose administered was considered as a toxic dose. However, if the mortality was observed only one mouse out of three animals then the same dose was repeated again to confirm the toxic effect. If mortality was not observed, the procedure was then repeated with higher dose (Organization for economic Co-operation and development, 2001).[68]

Observations

Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours) and daily thereafter, for a total of 14 days. All observations were systematically recorded with individual records being maintained for each animal. Observations included changes in skin, mortality and general behavioral pattern. Attention was given for observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. No death was observed till the end of study.

Acute Toxicity Record Sheet of *Phyllanthus urinaria*

Title: Evaluation of LD_{50} ***Phyllanthus urinaria***

Drug*: Phyllanthus urinaria* Dose: 2000 mg/kg BW Species: Albino mice Sex: Male & Female

Date: Duration: 24 hours

S.no.	**Code**	**Toxicity**		**Time Of Death**	**Observation**										
		Onset	**Stop**		**Skin colour**	**Eyes**	**Resp**	**CNS**	**Tre**	**Con**	**Sali**	**Diah**	**Slep**	**Leth**	**Com**
1.	**EPU**	x	x	X	x	x	x	x	X	X	x	x	x	x	X

(*TRE-Tremor, CON-Convulsions, SALI- Salivation, Diah - Diarrhea, LET-Lethargy) **x** = Negative, ✔ = Positive

EPU: Ethanolic extract of *Phyllanthus urinaria*

Qualitative chemical test:[69]

Preliminary phytochemical studies: Ethanolic extract of the plant of *Phyllanthus urinaria* were subjected to chemical tests for the identification of their active constituents.

1. **Tests for carbohydrates and glycosides**

A small quantity of the extract was dissolved separately in 4 ml of distilled water and filtered. The filtrate was subjected to Molisch's test to detect the presence of carbohydrates.

A. Molisch's Test

Filtrate was treated with 2-3 drops of 1% alcoholic α-naphthol solution and 2ml of con. H2SO4 was added along the sides of the test tube. Appearance of violet coloured ring at the junction of two liquids shows the presence of carbohydrates. Another portion of the extract was hydrolysed with HCl for few hours on a water bath and the hydrolysate was subjected to Legal's and Borntrager's test to detect the presence of different glycosides.

B. Legal's Test

To the hydrolysate, 1ml of pyridine and few drops of sodium nitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

C. Borntrager's Test : Hydrolysate was treated with chloroform and then the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Ammoniacal layer acquires pink colour showing the presence of glycosides.

2. Tests for alkaloids: A small portion of the methanol extract was stirred separately with few drops of dil. HCl and filtered. The filtrate was treated with various reagents as shown for the presence of alkaloids.

Mayer's reagent	-	Creamy precipitate
Dragandroff's reagent	-	Orange brown precipitate
Hager's reagent	-	Yellow precipitate
Wagner's reagent	-	Reddish brown precipitate

3. Tests for phytosterol

The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol.

Libermann Burchard Test

The residue was dissolved in few drops of acetic acid, 3 drops of acetic anhydride was added followed by few drops of con. H2SO4. Appearance of bluish green colour shows the presence of phytosterol.

4. Tests for fixed oils

Spot test

Small quantity of extract was separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oil. Few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 1-2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

5. Tests for gums and mucilages

Small quantity of the extract was added separately to 25 ml of absolute alcohol with constant stirring and filtered. The precipitate was dried in air and examined for its swelling properties for the presence of gums and mucilages.

6. Tests for Saponins

The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam shows the presence of saponins.

7.Tests for proteins and free amino acids

Small quantity of the extract was dissolved in few ml of water and treated with following reagents.

A. Millon's reage Appearance of red colour shows the presence of protein and free amino acids.

B. Ninhydrin reagent - Appearance of purple color shows the presence of proteins and free amino acids.

C. Biuret test - Equal volumes of 5% NaOH solution and 1% copper sulphate solution were added. Appearance of pink or purple colour shows the presence of proteins and free amino acids.

8.Tests for phenolic compounds and tannins

Small quantity of the extract was taken separately in water and tested for the presence of phenolic compounds and tannins using following reagents.

A. Dil.FeCl3 solution (5%)	-violet colour
B. 1% solution of gelatin containing 10% NaCl	- white precipitate
C. 10% lead acetate solution	- white precipitate.

9. Tests for flavonoids

A. With aqueous Sodium hydroxide solution:

Blue to violet colour (anthocyanins), yellow colour (flavones), yellow to orange (flavonones)

B. With Con. H2SO4:

Yellow orange colour (anthocyanins), yellow toorange colour (flavones), orange to crimson (flavonones)

C. Shinoda's test

Small quantity of the extract was dissolved in alcohol and to that a piece of magnesium followed by Con. HCl drop wise was added and heated. Appearance of magenta colour shows the presence of flavonoids.

The results of preliminary phytochemical studies of the plant extract are presented.

PHARMACOLOGICAL SCREENING MODEL

Ethylene glycol & ammonium chloride induced urolithiasis model[12]

Thirty healthy adult Wistar albino strain rats of either sex weighing 180-220g were randomly divided into five groups. Each group consisted of 6 animals. The treatment period was considered for 10 days.

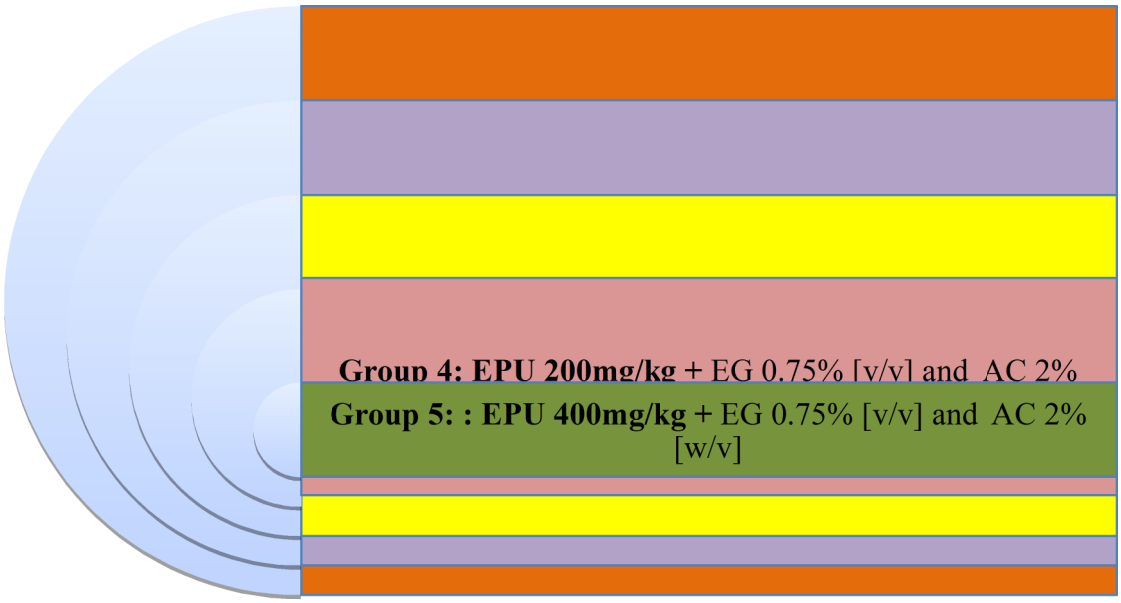
Group 1: Normal rats were fed with standard rat chow diet and tap water ad libitum for 10 days.

Group 2: EG and ammonium chloride intoxicated rats were given normal lab diet + drinking water containing 0.75% [v/v] ethylene glycol (EG) and 2% [w/v] ammonium chloride (AC) for 10 days to induce urolithiasis.

Group 3: Standard group were fed with normal lab diet + drinking water containing 0.75% [v/v] EG and 2% [w/v] AC + Cystone (5 ml/kg) for 10 days.

Group 4: the test groups treated with ethanolic extract *Phyllanthus urinaria* 200 mg/kg with normal lab diet + drinking water containing 0.75% [v/v] EG and AC 2% [w/v].

Group 5: the test groups treated with ethanolic extract *Phyllanthus urinaria* 400 mg/kg of body weight were fed with normal lab diet + drinking water containing 0.75% [v/v] EG and AC 2% [w/v].



URINE AND BLOOD SAMPLING

The crystalluria and stone formation was verified by different biochemical marker analysis of urine and serum. The urine samples of the test animals in different groups were collected in their respective end day of the experiment (1%) EG model on 10th day in (0.75%) EG + (2%) AC model. The collected urine sample volume and PH were measured followed by centrifugation at 3000 rpm for 10 minutes. After centrifugation

the urine samples were examined under light microscope (LAICA, DME Germany 400X) to ensure the presence of oxalate microcrystal followed by biochemical analysis (urine oxalate, calcium and uric acid, creatinine, urea, magnesium and phosphorus). The blood samples were collected from the animals under anaesthesia (ether) before sacrificing. The collected blood samples were then centrifuged to obtain serum for the analysis of serum creatinine and serum calcium, creatinine, urea, uric acid, magnesium and phosphorus.

Estimation of serum and urine parameters:

SERUM CREATININE (Mod. Jaffe's kinetic method)[62]

Principle

Picric acid in alkaline medium reacts with creatinine to form a orange coloured complex with the alkaline picrate. Intensity of colour formed during the fixed time measured at 520 nm is directly proportional to the amount of creatinine present in sample.

Creatinine + alkaline picrate ⟶ Creatinine picrate complex

Pipette in to test tubes	**Standard**	**Sample**
Working reagent	1000 µl	1000µl
Standard	100 µl	……..
Sample	……	100 µl

Mix and read the variation of absorbance (ΔA) between 30 seconds and 90 seconds

Calculation :

With standard or calibrator

$$\text{Concentration in sample (mg/dl)} = \frac{\text{Concentration of Standard}}{\text{Sample}} \times \frac{\Delta A}{\Delta A \text{ Standard}}$$

URIC ACID[63]

Principle:

Uric acid is oxidized to allantoin by uricase. The generated hydrogen peroxide reacts with 4-aminoantipyrine and DHPS to quinoneimine.

Uricase

Uric acid + H2O + O2 $\longrightarrow$ Allantoin + CO2 + H_2O_2

DHPS + 4-Aminoantipyrine + 2 H_2O_2 $\longrightarrow$ Quinoneimine + 3 H_2O

Assay procedure

Pipette in to test tubes	**Blank**	**Standard**	**Sample**
Working reagent 1	1000 µl	1000 µl	1000µl
Distill water	25µl	……	……..
Standard	……	25 µl	……..
Sample	…….	……	25 µl

Mix and read the absorbance (A) after a 10 minutes incubation but within 30 minutes.

Calculation :

With standard or calibrator

$$\text{Conc. of unknown Sample} = \frac{\text{Concentration of Standard}}{\text{Abs. Standard - Abs. of Reagent Blank}} \times \text{Abs. of unknown Sample} - \text{Abs.of Reagent Blank}$$

UREA[64]

Principle

Enzymatic determination according to the following reactions :

$$\text{Urea} + 2H\,O \xrightarrow{\text{Urease}} 2NH_4^+ + Co_3^{-2}$$

$$NH_4^+ + \text{Tris Base} + NADH \xrightarrow{\text{GLDH}} \text{L-Glu} + NAD^+ + H_2O$$

ASSAY PROCEDURE: Two Reagent Procedure

	Standard	**Sample / Control**
R1	800 µl	800 µl
R2	200 µl	200 µl

Mix and Incubate at 37°C for 2 minutes then add

Pipette in to test tubes	Standard	Sample
Working reagent	1000 µl	1000µl
Standard	10µl	……..
Sample	……	10µl

Mix and read the variation of absorbance (ΔA) between 30 seconds and 60 seconds.

Calculation :

With standard or calibrator

$$\text{Concentration in Sample (mg/dl)} = \frac{\text{Concentration of Standard}}{\Delta A \text{ Standard}} \times \Delta A \text{ Sample}$$

CALCIUM (OCPC)

Principle :

Ortho-Cresolphthalein Complexone reacts with calcium ions in alkaline medium forming a red-violet color. Interference by magnesium is eliminated by addition of 8-hydroxyquinoline. The colour intensity is directly proportional to the serum total calcium concentration.

Assay procedure:

Pipette in to test tubes	Blank	Standard	Sample
Working reagent 1	1000 µl	1000 µl	1000µl
Distill water	10µl	……	……..
Standard	……	10µl	……..
Sample	…….	……	10 µl

Mix and read the absorbance (A) after 5 minutes incubation, but within 30 minutes.

Calculation :

With standard or calibrator.

$$\text{Conc. of unknown Sample} = \frac{\text{Concentration of Standard}}{\text{Abs. Standard} - \text{Abs. of Reagent Blank}} \times \text{Abs. of unknown Sample} - \text{Abs. of Reagent Blank}$$

PHOSPHORUS

Principle :

Ammonium molybdate + Sulfuric Acid ——— Phosphomolybdate complex

Assay procedure :

Pipette in to test tubes	Blank	Standard	Sample
Working reagent 1	1000 µl	1000 µl	1000µl
Distill water	10µl	……	……..
Standard	……	10µl	……..
Sample	…….	……	10 µl

Mix and read the absorbance (A) after a 5 minutes incubation.

Calculation :

With standard or calibrator.

$$\text{Conc. of unknown Sample} = \frac{\text{Concentration of Standard}}{\text{Abs. Standard} - \text{Abs. of Reagent Blank}} \times (\text{Abs. of unknown Sample} - \text{Abs. of Reagent Blank})$$

Oxalate

Principle:

Oxalate is co-precipitated with calcium sulphate, reduced to glycolic acid by boiling with dilute sulphuric acid and a zinc pellet and estimated colorimetrically with chromotropic acid.

Procedure:

Set the auto-analyzer instrument with the parameters given along with the kit. Prepare the working, standard and test solutions as per the protocol. Incubate for 5 min at room temperature mix and read on colorimeter.

Calculations:

$$\text{Oxalate (mg/dl)} = \frac{\text{Absorbance of Test} \times \text{Concemtration of Standard (mg/dl)}}{\text{Absorbance of standard}}$$

MAGNESIUM

Principle:

Magnesium combines with calmagite in an alkaline medium to form a red coloured complex. Interference of calcium and proteins is eliminated by the addition of specific

chelating agent and detergents, Intensity of the colour formed is directly proportional to the amount of magnesium present in the sample.

Magnesium+Calmagite $\xrightarrow{\text{Alkaline mdeium}}$ Red coloured complex

Procedure:

Set the auto-analyzer instrument with the parameters given along with the kit. Prepare the working standard and test solutions as per the protocol. Incubate for 5 min at room temp. Mix and read 510 nm within 30 min.

Calculations:

$$\text{Magnesium mEq/L} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times 2$$

HISTOPATHOLOGY

The abdomen was cut open to remove both kidneys from each animal. Isolated kidneys were rinsed in an ice-cold physiological solution, after the extraneous tissues were removed. The right kidney was fixed in 10% neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 μm and stained with hematoxylin and eosin (H and E) for histopathological examination. The slides were examined under a light microscope to study the architecture of the kidney and calcium oxalate deposits.

ENZYME ASSAY

A portion of kidney was taken from all the groups, and a 30% w/v homogenate was prepared in 0.9% buffered KCl (pH 7.4) for the estimation of protein, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA).

1. Lipid Peroxidation:

Malondialdehye, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehye reacts with thiobarbituric acid (TBA) to produce red colored species which is measured at 532 nm.

Reagents:

1. TBA-TCA-HCl reagent.

15% w/v TCA, 0.375 %w/v TBA and 0.25 N HCl. This solution was mildly heated to assist the dissolution of TBA

1 ml of kidney homogenate was combined with 2 ml of TCA-TBA-HCl reagent and mixed thoroughly. The solution was heated for 15 min. in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. the absorbance of the supernatant was measured at 532 nm against a blank that contains all the reagents minus the kidney homogenate. The malondialdehyde concentration of the sample can be calculated using an extinction coefficient of $1.56x10^5 M^{-1}cm^{-1}$

$$\text{Malondialdehyde concentration (M)} = \frac{\text{Absorbance}}{1.56 \times 10^5}$$

2. Estimation of SOD

The activity of superoxide dismutase was determined by the method of Misra and Fridovich (1972) based upon the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH inhibition of the chromagen formation by superoxide dismutase was linear with increase in enzyme concentration.

Reagents:

1. Sodium carbonate buffer 0.1M (pH 10.2)
2. Ephinephrine(1mM)

The entire supernatant 1 ml (S) was taken in 0.1 M carbonate buffer (pH 10.2). After addition of epinephrine, the increase in absorbance was measured at 480 nm using a UV–Visible double beam spectrophotometer. The activity of the enzyme has been expressed as U/mg protein, where 1U of the enzyme is defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50% under the conditions of the assay.

3. Estimation of Catalase

In the presence of catalase, H_2O_2 shows a continual decrease in absorbane in UV range. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240nm (E_{240} = 0.00394 ±0.0002 ltr $mmol^{-1}$ mm^{-1}). The difference in absorbance (Δ A 240) per unit time is a measure of the catalase activity.

Reagents:

1. PBS 50 mM; pH7.0

Dissolve (a)6.81 of KH2PO4 and (b) in the proportion 1:1.5(v/v).

2. H_2O_2 (0.17 mM): dilute 0.16 ml of (30%w/v) H_2O_2 with Phosphate buffer to 100 ml.

The catalase activity was determined spectrophotometrically according to the protocol of Claiborne (1985).The reaction mixture (2 ml) contained 1.95 ml 10 mM H2O2 in 60 mM phosphate buffer (pH 7.0).The reaction was started by adding 0.05 ml supernatant and the absorbance was followed for 3 min at 240 nm. Phosphate buffer (60 mM, pH 7.0) was used as a reference. The extinction coefficient of 0.04 $mM^{-1}cm^{-1}$ was used to determine the specific activity of catalase. A unit of catalase is defined as the quantity, which decomposes 1.0µmole of H_2O_2 per min at pH 7.0 at 25^0C, while this H_2O_2 concentration falls from 10.3to 9.2mM.The data was expressed as U/mg protein

4. Estimation of glutathione (GSH):

Principle: GSH is a non protein compound containing sulphydryl group in its structure. DTNB (5,5' di thio bis (2-Nitrobenzoic acid) is a disulfide chromagen that is reduced by sulphydryl compounds to an intensly yellow colored compound. The absorbance of the reduced chromagen is measured at 412 nm and is directly proportional to the GSH concentration.

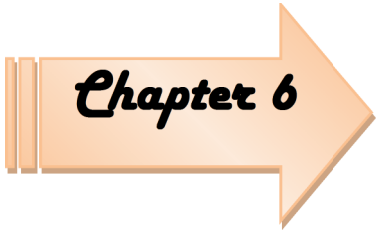
Reagents:

1. 10% Trichloroacetic acid (TCA)
2. Phosphate buffer (0.2M) pH 8.0
   0.218 g Sodium dihydrogen phosphate and 2.641 g disodium hydrogen phosphate in 100 ml distilled water
3. DTNB (0.6 mM) (pH 8)
   11.9 mg in 50 ml Phosphate buffer

GSH was measured by the method of Moran et al(1979).The kidney homogenate proteins were precipitated by 10% TCA, centrifuged and the supernatant was collected. 1 ml of supernatant was mixed with 6 ml of 0.2 M Phosphate buffer pH 8.0 and 1 ml 0.6 mM DTNB and incubated for 10 min at room temperature. The absorbance of the samples was recorded against the blank at 412 nm and the GSH concentration was calculated from the standard curve by multiplying with the dilution factor (mannervik1985; tetza 1969)

Statistical analysis

Results were indicated in terms of mean ± SEM. Statistical significance of data were assessed by analysis of variance (One way-ANOVA), followed by comparison between different groups using 'Dunnett's multiple comparison test. The significance was considered at the level of $P<0.05$.



RESULTS

6.RESULTS

Preliminary phytochemical screening

The extract of drug were analysed for the presence of various constituents. The result of this preliminary phytochemical examination is shown in Table no.

Table No. Qualitative chemical examination of ethanolic extract of *Phyllanthus urinaria.*

Phytoconstituents	Presence or Absence
Carbohydrates	+
Glycosides	+

Fixed oils and fats	+
Gums & mucilage	-
Potein & amino acids	-
Saponins	++
Tannins	+
Phytosterols	+
Flavonoids	+++
Alkaloids	+++

PHARMACOLOGICAL ACTIVITY

Acute oral toxicity

Acute oral toxicity was carried out according to OECD guideline. EPU was safe upto 2000mg/kg.

Effect of ethanolic extract of *Phyllanthus urinaria* on urinary volume and pH against EG and AC induced urolithiasis.

EG and AC (0.75 and 2%.) administration showed significant ($p<0.001$) alteration of the output of urine and pH as compared normal group. Administration of Cystone 5ml/kg, EPU 200 and 400 mg/kg caused significamtly increased ($p<0.01$, $p<0.05$) urine output and pH of the urine as compared to control (EG and AC) group.

Effect of ethanolic extract of *Phyllanthus urinaria* on serum biochemical parameters against EG and AC induced urolithiasis.

Serum Creatinine

Administration of EG and AC (0.75% and 2%) for 10 days caused significant elevation ($p<0.01$) in serum creatinine concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in serum creatinine concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.05$ and $p<0.001$) in serum creatinine concentration when compared to EG and AC alone treated group.

Serum Urea

Administration of EG and AC (0.75% and 2%) for 10 days caused significant elevation ($p<0.001$) in serum urea concentration compared to normal one. Standrad cystone 5ml/kg

causes significant reduction ($p<0.001$) in serum urea concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.01$ and $p<0.01$) in serum urea concentration when compared to EG and AC alone treated group.

Serum Uric Acid

Administration of EG and AC (0.75% and 2%) for 10 days caused significant elevation ($p<0.01$) in serum uric acid concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in serum uric acid concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.05$ and $p<0.001$) in serum uric acid concentration when compared to EG and AC alone treated group.

Serum Calcium

Administration of EG and AC (0.75% and 2%) for 10 days caused significant elevation ($p<0.001$) in serum calcium concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in serum calcium concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.001$ and $p<0.001$) in serum calcium concentration when compared to EG and AC alone treated group.

Serum Oxalate

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in serum oxalate concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in serum oxalate concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.05$ and $p<0.001$) in serum oxalate concentration when compared to EG and AC alone treated group.

Serum Phosphorus

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in serum phosphorus concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in serum phosphorus concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.05$ and $p<0.001$) in serum phosphorus concentration when compared to EG and AC alone treated group.

Serum Magnesium

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.05$) in serum magnesium concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.01$) in serum magnesium concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.01$ and $p<0.001$) in serum magnesium concentration when compared to EG and AC alone treated group.

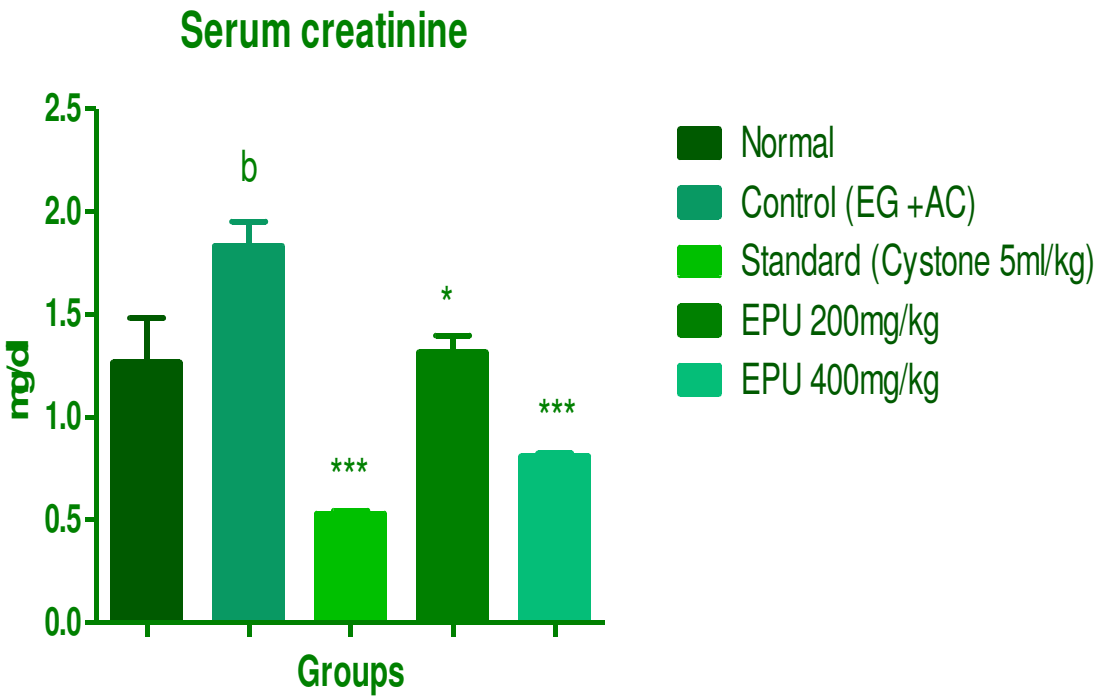
Treatment group	**Urinary Volume (ml/24hr)**	**Urine Ph**
Normal	18.4±0.97	7.5±2.1
Control (EG+AC)	6.96±0.69a	4.5±1.39a
Standard Cystone (5ml/kg)	14.93±0.57***	8.2±1.32**
EPU 200mg/kg	8.41±0.45*	5.9±1.21*
EPU 400mg/kg	9.9±0.64*	6.14±2.24**

Treatment group	Serum Biochemical Parameters						
	Creatinine mg/dl	**Urea mg/dl**	**Uric Acid mg/dl**	**Calcium mg/dl**	**Oxalate mg/dl**	**Phosphorus mg/dl**	**Magnesium mg/dl**
Normal	1.26±0.21	39.48±0.50	5.720±0.02	2.58±0.03	1.88±0.24	3.24±0.26	1.85±0.19
Control (EG+AC)	1.83±0.12b	48.94±0.57a	18.64±0.04a	6.25±0.25a	3.73±0.28a	5.48±0.29a	2.72±0.13c
Standard Cystone (5ml/kg)	0.53±0.01***	41.99±0.41***	10.82±0.03***	2.87±0.10***	1.99±0.21***	3.07±0.29***	1.73±0.20**
EPU 200mg/kg	1.31±0.08*	51.69±0.50**	10.26±0.08***	4.517±0.20***	2.65±0.31*	4.16±0.46*	1.79±0.18**
EPU 400mg/kg	0.81±0.01***	39.28±0.49**	8.55±0.03***	2.99±0.10***	1.66±0.23***	3.36±0.19***	1.58±0.20**

Table.no.1: Effect of ethanolic extract of *Phyllanthus urinaria* on serum parameters against EG and AC induced urolithiasis

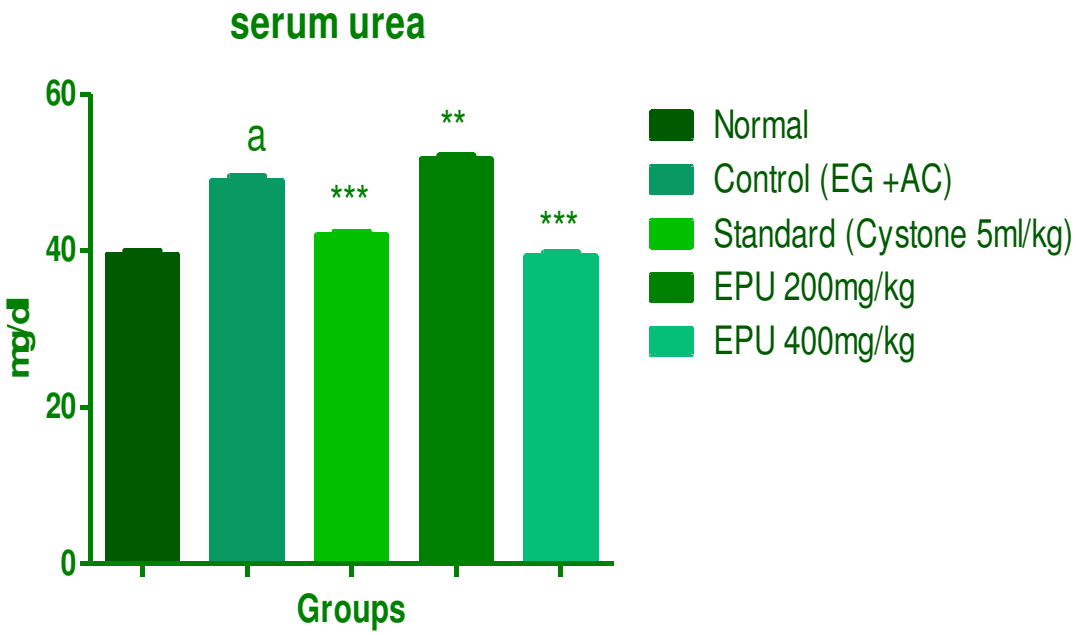
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, **p<0.01, ***p<0.001 as compared to control and [a]p<0.001,bp<0.01 and p<0.05 as when compared to normal.

Fig.no.1: Effect of ethanolic extract of *Phyllanthus urinaria* on serum creatinine against EG and AC induced urolithiasis



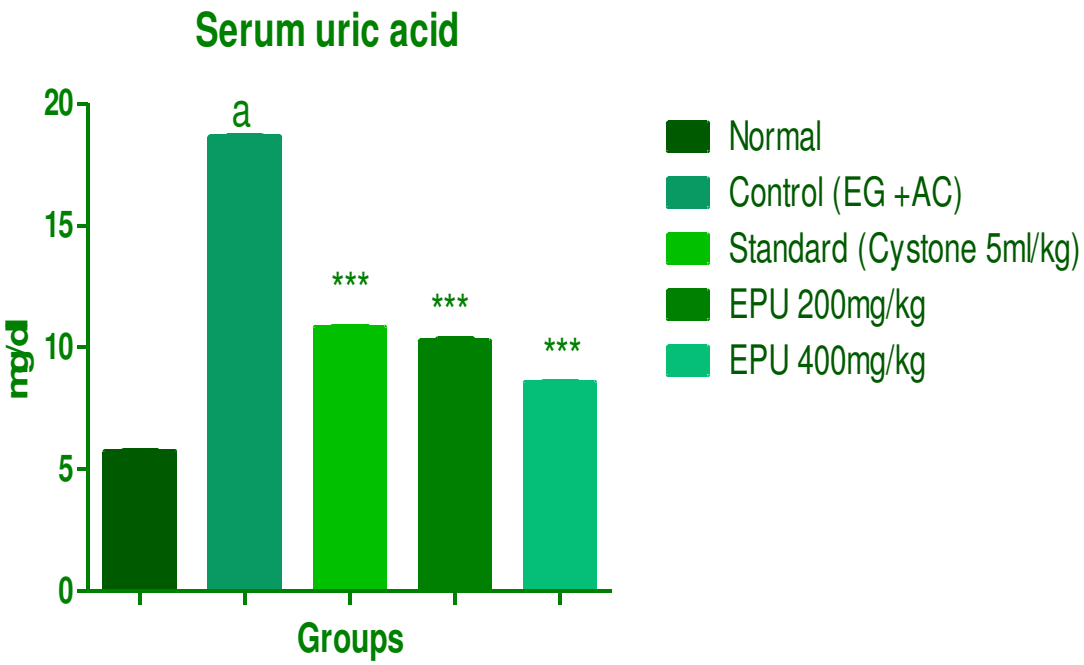
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, ***p<0.001 as compared to control and bp<0.01 as when compared to normal.

Fig.no.2: Effect of ethanolic extract of *Phyllanthus urinaria* on serum urea against EG and AC induced urolithiasis.



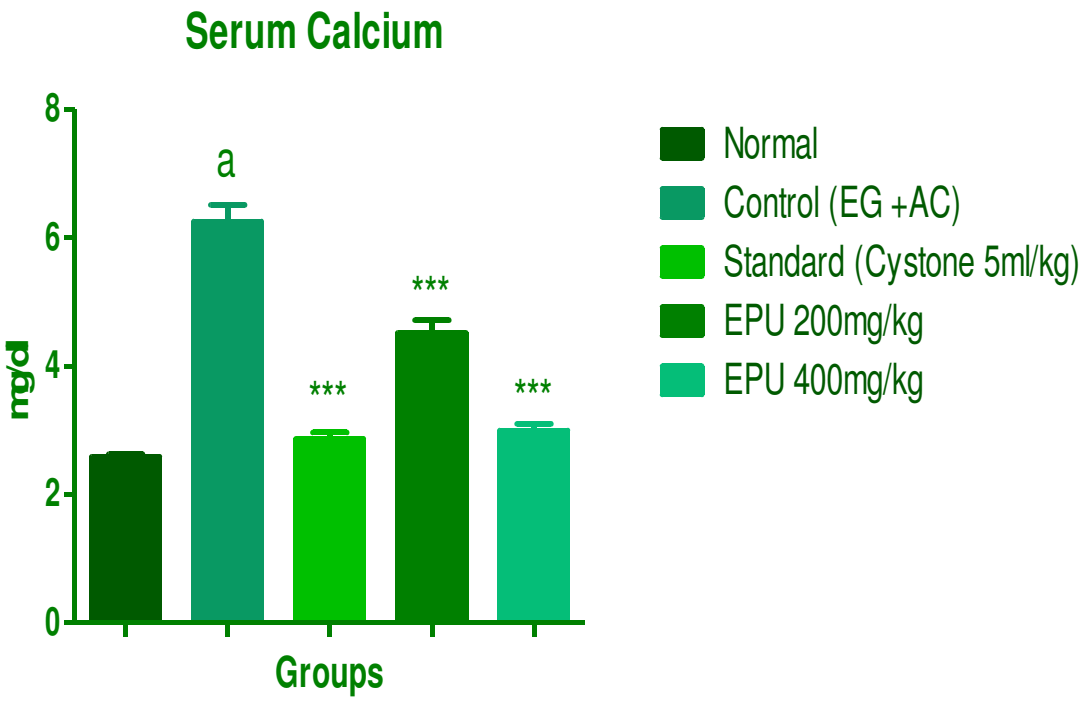
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, **p<0.01, ***p<0.001 as compared to control and [a]p<0.001as when compared to normal.

Fig.no.3: Effect of ethanolic extract of *Phyllanthus urinaria* on serum uric acid against EG and AC induced urolithiasis.



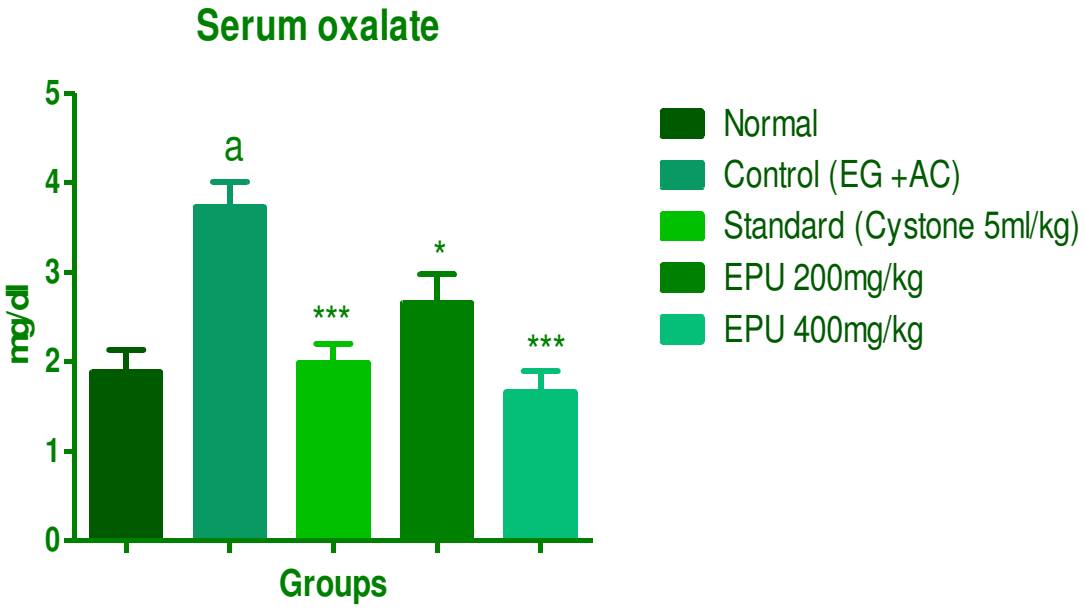
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.4: Effect of ethanolic extract of *Phyllanthus urinaria* on serum calcium against EG and AC induced urolithiasis.



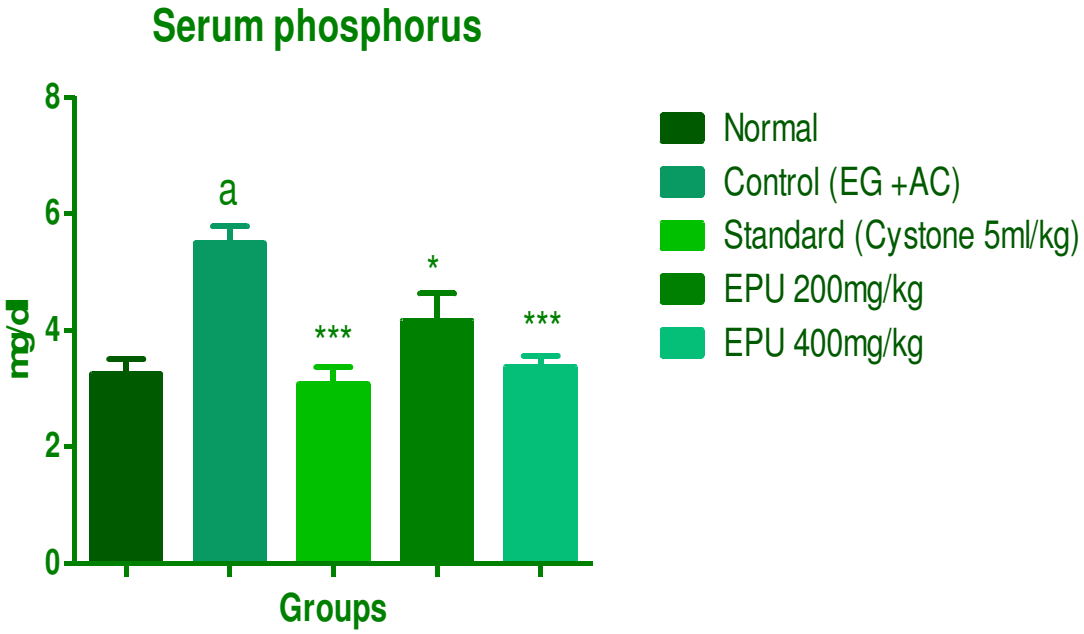
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, ***p<0.001 as compared to control and [a]p<0.001 as when compared to normal.

Fig.no.5: Effect of ethanolic extract of *Phyllanthus urinaria* on serum oxalate against EG and AC induced urolithiasis.



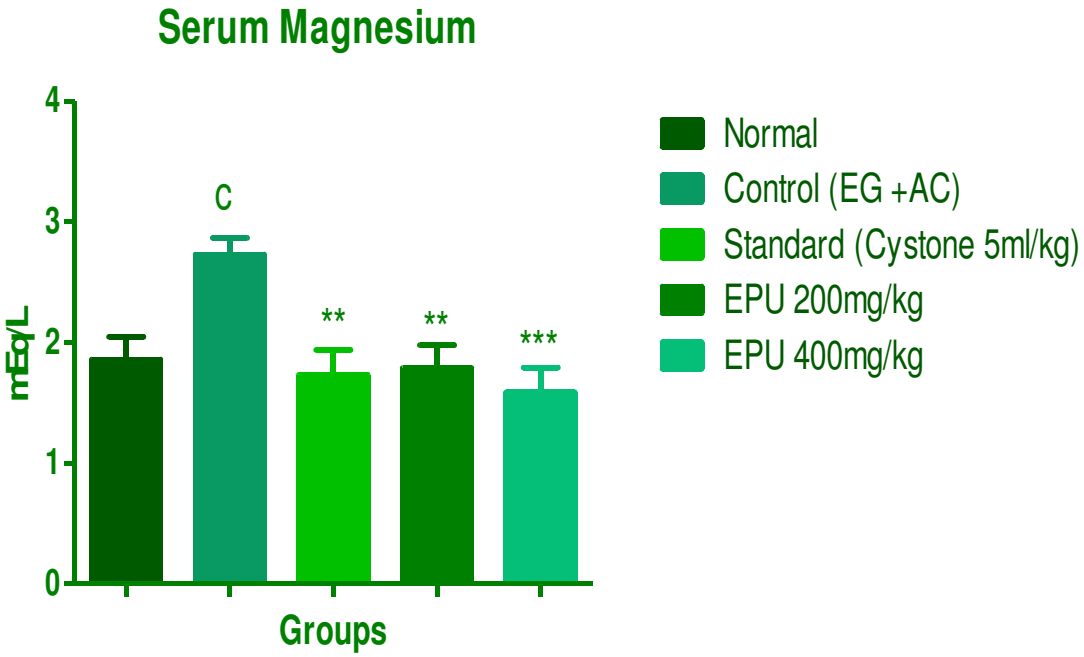
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, ***p<0.001 as compared to control and [a]p<0.001 as when compared to normal.

Fig.no.6: Effect of ethanolic extract of *Phyllanthus urinaria* on serum phosphorus against EG and AC induced urolithiasis.



All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, ***p<0.001 as compared to control and [a]p<0.001 as when compared to normal.

Fig.no.7: Effect of ethanolic extract of *Phyllanthus urinaria* on serum creatinine against EG and AC induced urolithiasis.



All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test,**p<0.01, ***p<0.001 as compared to control and cp<0.05 as when compared to normal.

Effect of ethanolic extract of *Phyllanthus urinaria* on urine biochemical parameters against EG and AC induced urolithiasis.

Urinary Creatinine:

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in urine creatinine concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.01$) in urine creatinine concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction (not significant and $p<0.001$) in urine creatinine concentration when compared to EG and AC alone treated group.

Urinary urea

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in urine urea concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in urine urea concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.01$ and $p<0.001$) in urine urea concentration when compared to EG and AC alone treated group.

Urinary Calcium

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in urine calcium concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in urine calcium concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.05$ and $p<0.001$) in urine calcium concentration when compared to EG and AC alone treated group.

Urinary Oxalate

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in urine oxalate concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in urine oxalate concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.001$ and $p<0.001$) in urine oxalate concentration when compared to EG and AC alone treated group.

Urinary Phosphorus

Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in urine phosphorus concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in urine phosphorus concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.05$ and $p<0.001$) in urine phosphorus concentration when compared to EG and AC alone treated group.

Urinary Magnesium

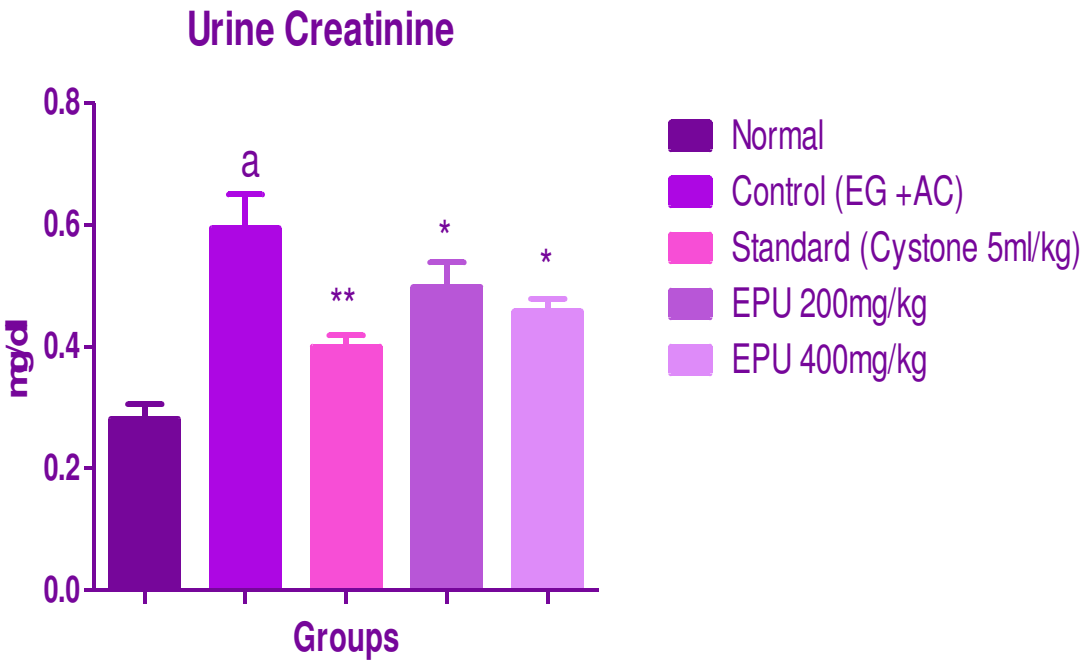
Administration of EG and AC (0.75% and 2%) for 10 days caused significant increased ($p<0.001$) in urine magnesium concentration compared to normal one. Standrad cystone 5ml/kg causes significant reduction ($p<0.001$) in urine magnesium concentration when compared to EG and AC alone treated group. Pretreatment with EPU 200 and 400mg/kg causes significant reduction ($p<0.05$ and $p<0.001$) in urine magnesium concentration when compared to EG and AC alone treated group.

Table.no.2: Effect of ethanolic extract of *Phyllanthus urinaria* on urine biochemical parameters against EG and AC induced urolithiasis.

Treatment group	Urine Biochemical Parameters						
	Creatinine mg/dl	**Urea mg/dl**	**Uric Acid mg/dl**	**Calcium mg/dl**	**Oxalate mg/dl**	**Phosphorus mg/dl**	**Magnesium mg/dl**
Normal	0.28±0.02	58.94±2.35	2.31±0.16	10.65±0.83	6.81±0.41	3.195±0.01	4.77±0.37
Control (EG+AC)	0.59±0.05a	88.82±3.19a	6.01±0.28a	18.49±1.03a	15.65±1.22a	9.093±0.46a	1.94±0.01a
Standard Cystone (5ml/kg)	0.40±0.01**	53.05±2.10** *	2.99±0.36***	11.62±0.72** *	7.03±0.46***	4.758±0.27** *	4.36±0.48** *
EPU 200mg/kg	0.49±0.04ns	73.48±3.53**	3.56±0.25***	14.37±0.98*	7.60±0.57***	7.58±0.45*	3.51±0.48*
EPU 400mg/kg	0.45±0.02*	64.88±2.10** *	3.44±0.23***	12.81±0.88** *	7.37±1.01***	5.94±0.31***	4.65±0.41** *

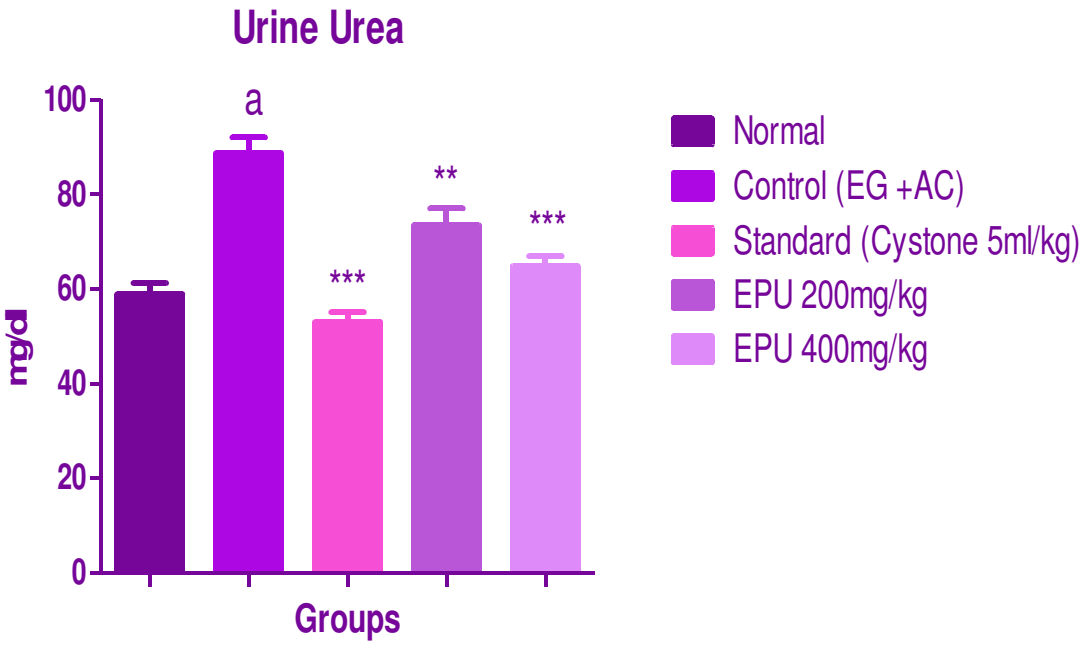
All the values are Mean±SEM, n=6, ns= not significant, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, **p<0.01, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.8: Effect of ethanolic extract of *Phyllanthus urinaria* on urine creatinine parameters against EG and AC induced urolithiasis.



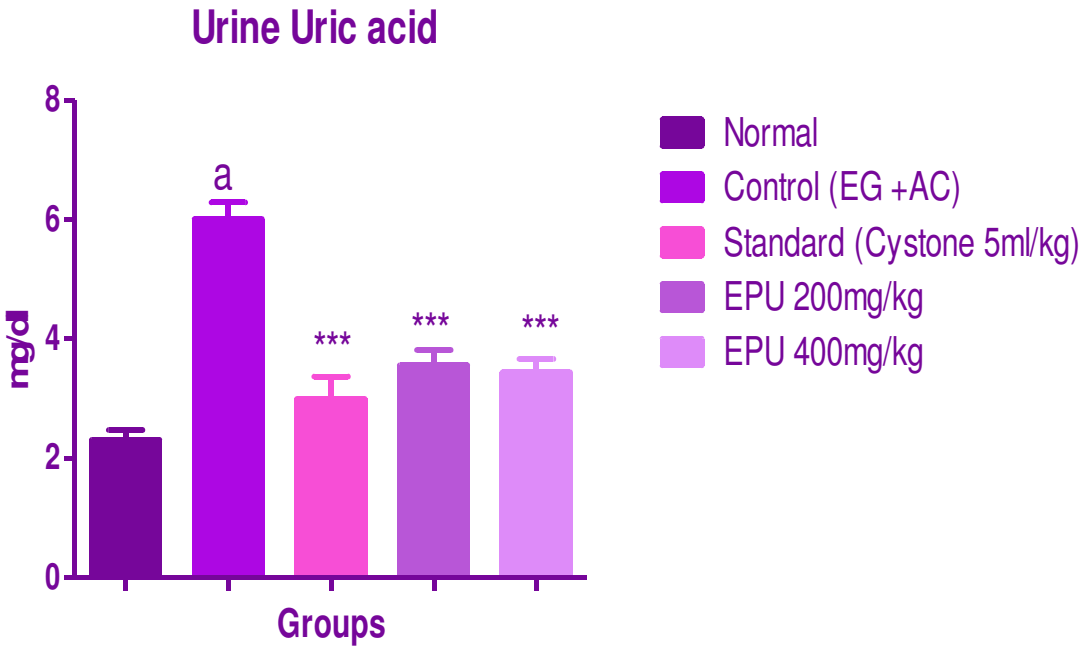
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparison of Dunnett's test, *p<0.05, **p<0.01 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.9: Effect of ethanolic extract of *Phyllanthus urinaria* on urine urea parameters against EG and AC induced urolithiasis.



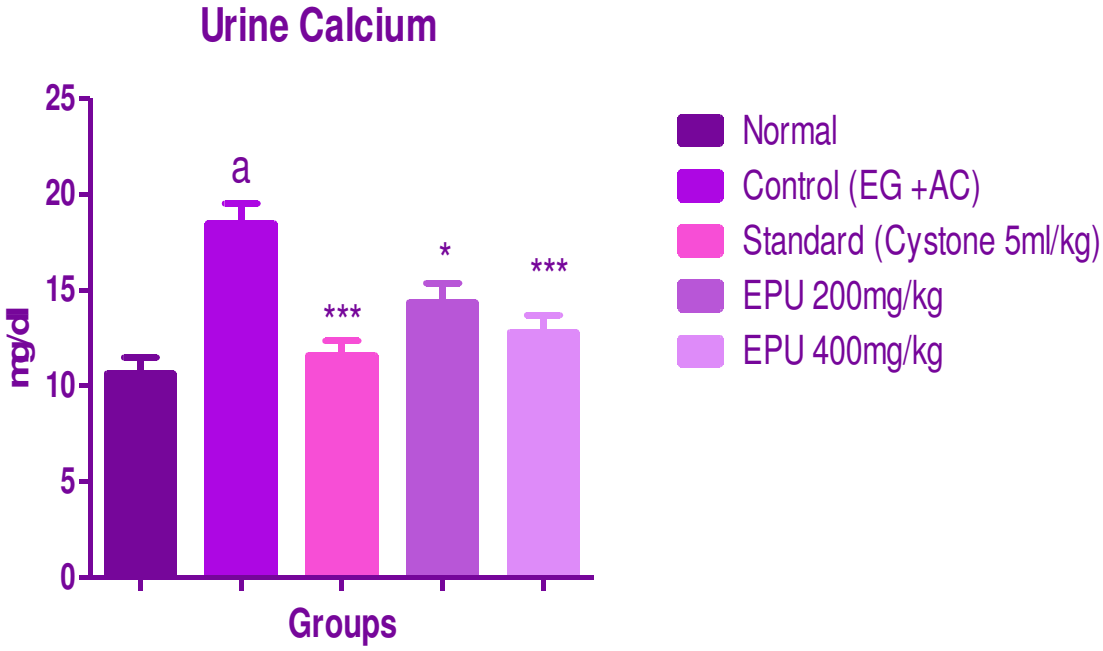
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparison of Dunnett's test, **p<0.01, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.10: Effect of ethanolic extract of *Phyllanthus urinaria* on urine uric aicd parameters against EG and AC induced urolithiasis.



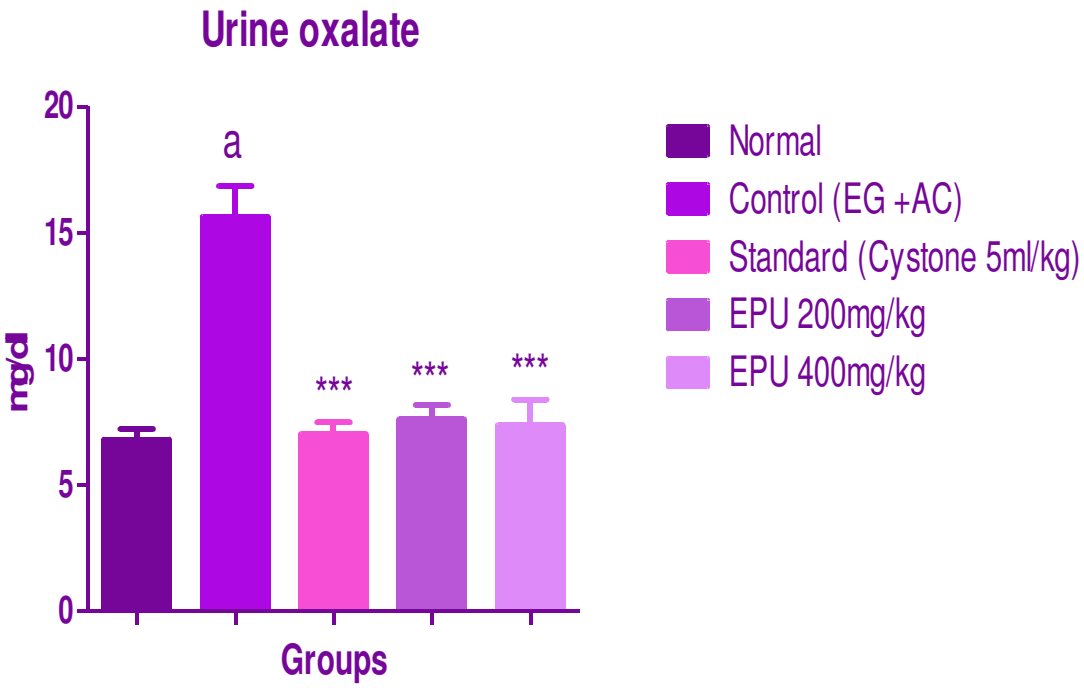
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparison of Dunnett's test, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.11: Effect of ethanolic extract of *Phyllanthus urinaria* on urine calcium parameters against EG and AC induced urolithiasis.



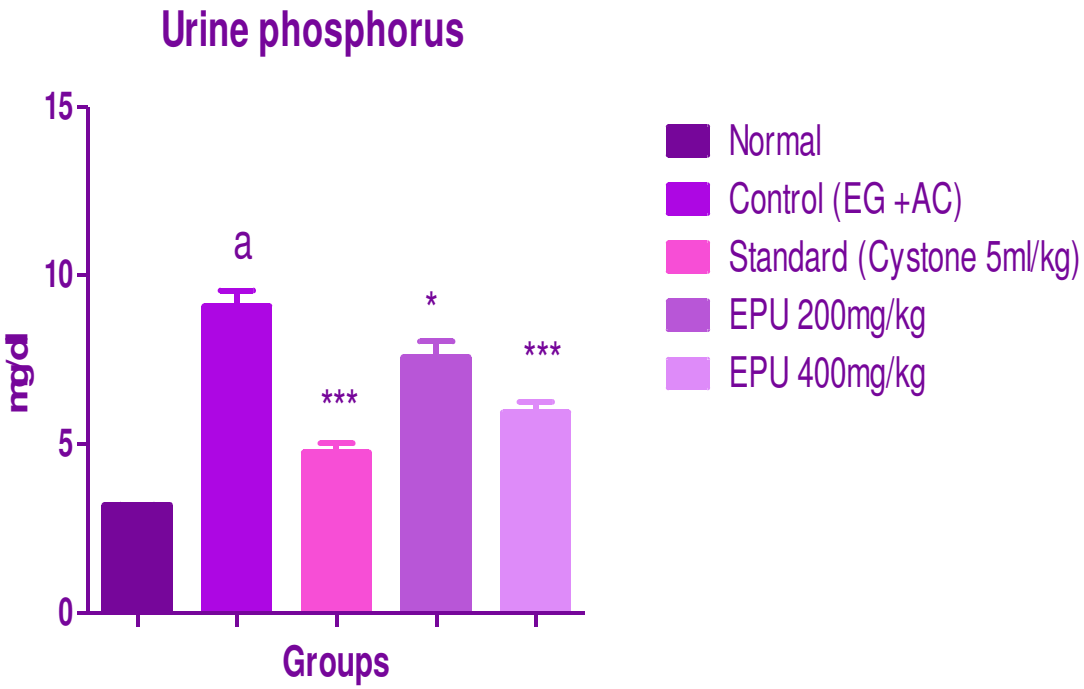
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparison of Dunnett's test, *p<0.05, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.12: Effect of ethanolic extract of *Phyllanthus urinaria* on urine oxalate parameters against EG and AC induced urolithiasis.



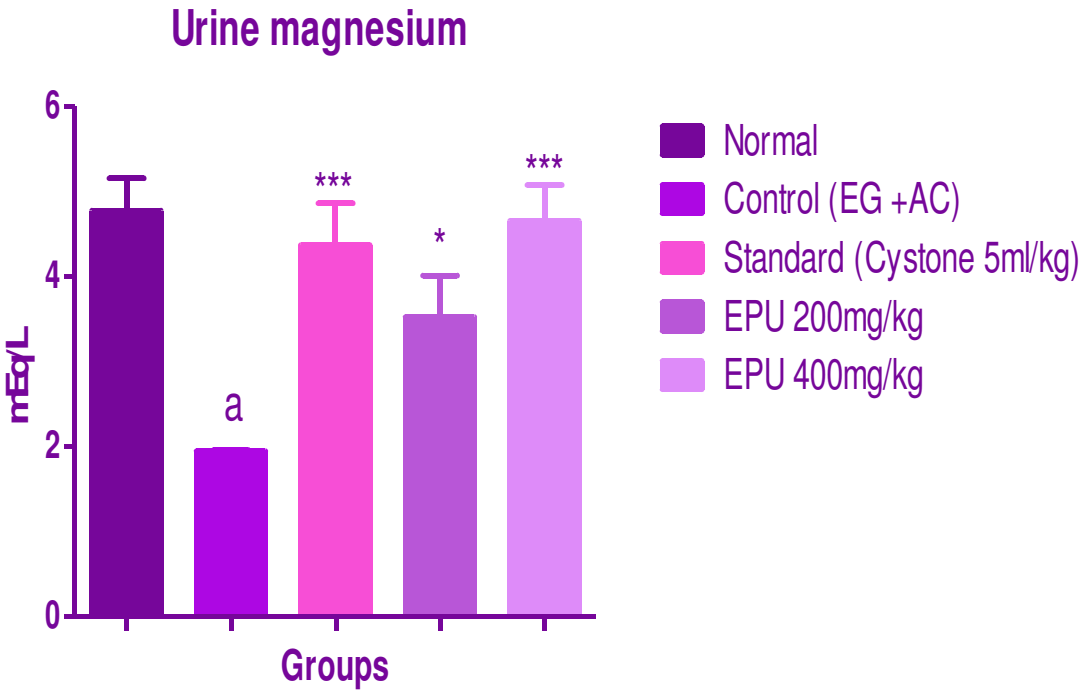
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparison of Dunnett's test, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.13: Effect of ethanolic extract of *Phyllanthus urinaria* on urine phosphorus parameters against EG and AC induced urolithiasis.



All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparison of Dunnett's test, *p<0.05, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.14: Effect of ethanolic extract of *Phyllanthus urinaria* on urine magnesium parameters against EG and AC induced urolithiasis.



All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Effect of ethanolic extract of *Phyllanthus urinaria* on LPO (kidney enzyme) parameters against EG and AC induced urolithiasis.

Oxidative stress

In-vivo LPO In control animals, EG/AC induced lithogenesis produced a significant enhancement in the renal MDA levels ($p<0.001$) respectively, when compared to the normal group. After treatment with standard cystone 5ml/kg and EPU 200 and 400mg/kg significant ($P<0.001$) reduction in the kidney MDA levels was observed in the treated groups, when compared to their respective control groups.

Catalase levels of the kidney were significantly ($p<0.001$) decreased in the control groups on EG/AC administration for 10 days, when compared to the normal group. On treatment with Standrad cystone and EPU 200 and 400mg/kg ($p<0.001$), a significant rise in the renal catalase levels was observed in treated groups (Table 3).

GSH levels of the kidney were significantly ($p<0.001$) decreased in the control groups on EG/AC administration for 10 days, when compared to the normal group. On treatment with Standrad cystone ($p<0.01$) and EPU 200 ($p<0.05$) and 400mg/kg

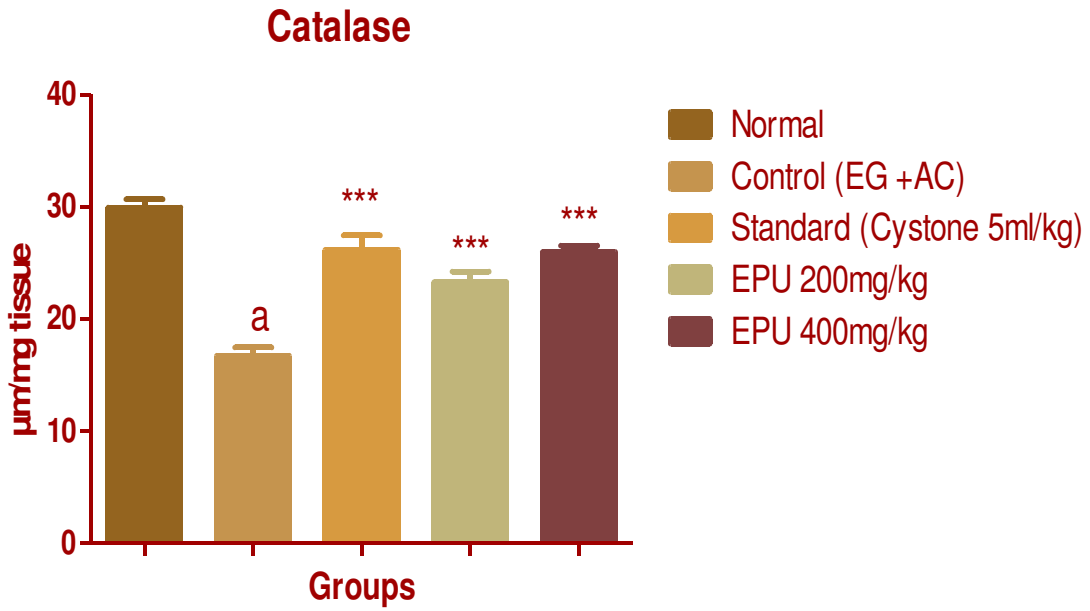
($p<0.01$), a significant rise in the renal catalase levels was observed in treated groups (Table 3)

Table.no.3: Effect of ethanolic extract of *Phyllanthus urinaria* on antioxidant (kidney enzyme) parameters against EG and AC induced urolithiasis.

Treatment group	Invivo Antioxidant parameters		
	CATALASA µm/mg tissue	**GSH µm/mg tissue**	**LPO µm of /H_2O_2/mg tissue**
Normal	29.95±0.72	14.97±0.67	133.1±3.60
Control (EG+AC)	16.73±0.74a	7.970±0.83a	219.8±4.65a
Standard Cystone (5ml/kg)	26.19±1.25***	12.80±0.68**	152.3±3.29***
EPU 200mg/kg	23.29±0.92***	9.74±1.04*	176.7±5.05***
EPU 400mg/kg	26.00±0.56***	12.68±0.85**	164.9±5.59***

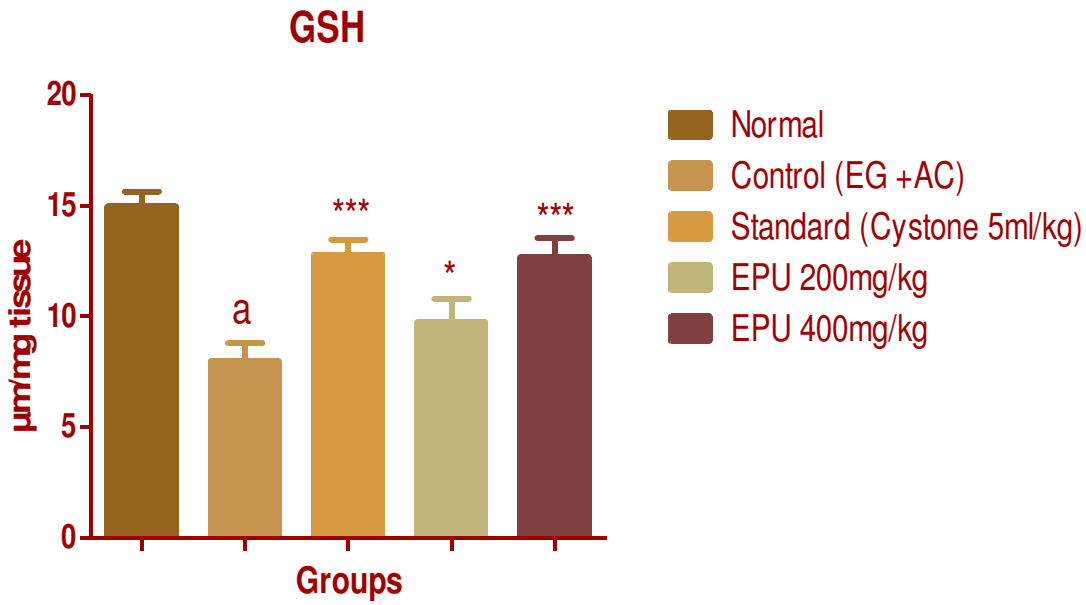
All the values are Mean±SEM, n=6, ns= not significant, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, **p<0.01, ***p<0.001 as compared to control and ^{a}p<0.001, as when compared to normal.

Fig.no.16: Effect of ethanolic extract of *Phyllanthus urinaria* on Catalase (kidney enzyme) parameters against EG and AC induced urolithiasis.



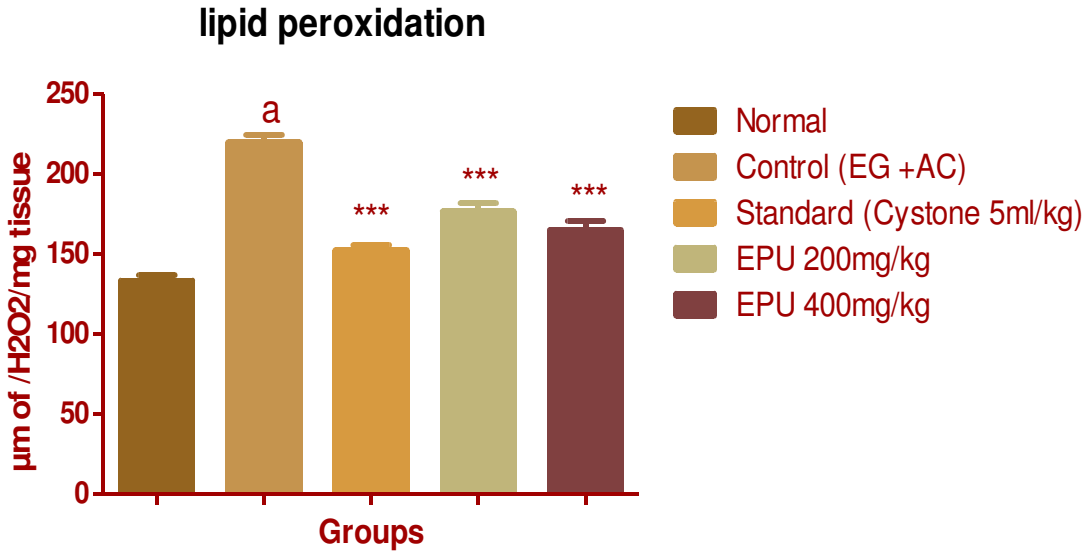
All the values are Mean±SEM, n=6, One way ANOVA followed by multiple comparison of Dunnett's test, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.17: Effect of ethanolic extract of *Phyllanthus urinaria* on Catalase (kidney enzyme) parameters against EG and AC induced urolithiasis.



All the values are Mean±SEM, One way ANOVA followed by multiple comparision of Dunnett's test, *p<0.05, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

Fig.no.18: Effect of ethanolic extract of *Phyllanthus urinaria* on Lipidperoxide (kidney enzyme) parameters against EG and AC induced urolithiasis.



All the values are Mean±SEM, One way ANOVA followed by multiple comparision of Dunnett's test, ***p<0.001 as compared to control and [a]p<0.001, as when compared to normal.

HISTOPATHOLOGY OF KIDNEY

Normal rats kidney showing normal cellular structure.

The histopathology of kidney samples of rats treated with EG+AC (0.75 and 2%) showed loss of normal architecture with presence of crystalline structure in dilated collecting tubules. The same section when viewed under polarising microscope revealed presence of white chalky coloured calcium oxalate crystals in several tubules and glomeruli. These groups also showed congestion of intersitum and inflammation of the pelvic calyceal systems.

The histopathology of kidney samples of rats treated with standard drug cystone 5ml/kg and EG+AC for 10 days showed normal architecture of the kidney.

The histopathology of kidney samples of rats treated with EUP 200mg/kg and EG+AC for 10 days showed mild colloidal cast inside tubules and EUP 400mg/kg showed cloudy changes and congestion of thes glomeruli. However the architecture of kidney approved almost normal.

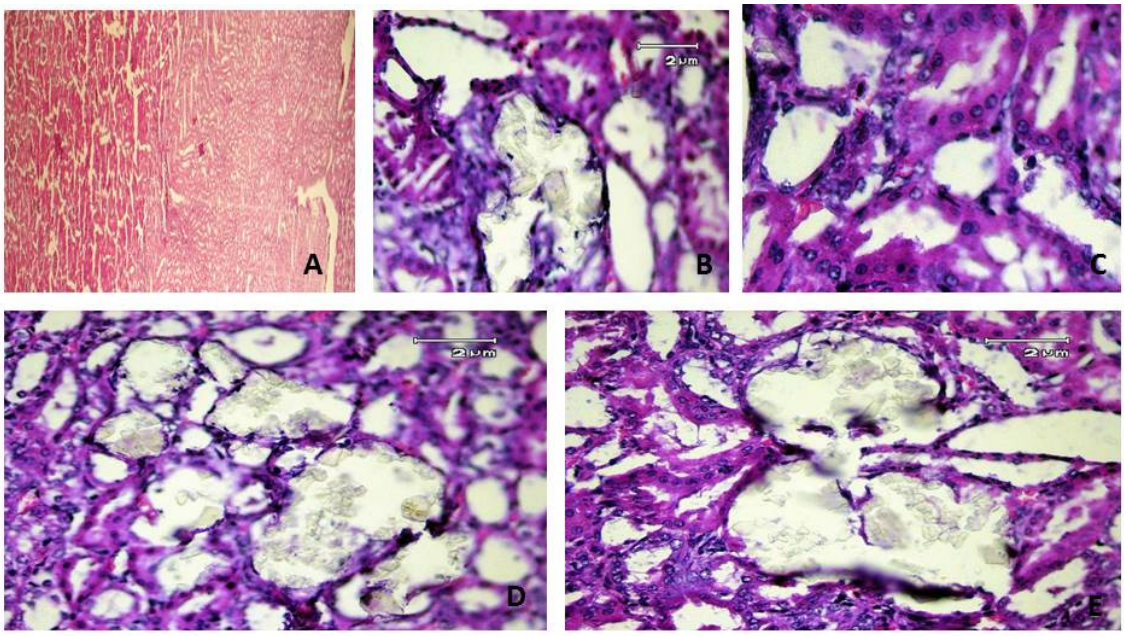
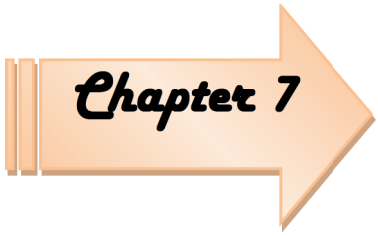


Fig.no.19: Histopathology of kidney

A: Normal group, B: EG+AC group:, C Standard (Cystone 5ml/kg) group, D group EPU 200mg/kg, E group EPU 400mg/kg,



7.DISCUSSION

Urinary stone disease is a common, painful and expensive medical condition[80]. Though extracorporeal shock wave lithotripsy has facilitated the stone removal and reduced the morbidity associated with urinary stone, recurrence is common[81]. Several experimental

and clinical studies on some of the plants used in the Indian traditional system of medicine proved their efficacy in the management of renal stone disease. Therefore, it is advisable to evaluate plants used in the traditional medicine to treat kidney stone disease for Antiurolithiatic activity, which might be also useful in reducing stone recurrence rate.

Rats are commonly used to study the pathogenesis of human CaOx kidney stone disease, as Ox metabolism is regarded almost similar in rats and humans[82]. Ingestion of EG/AC has been found to be a reliable inducer of Ox lithiasis in rats. EG is converted to endogenous oxalic acid by the liver enzyme glycolate oxidase [19] and AC induces urinary acidification, is supposed to upset the enzyme sorting mechanism in the tubular cells in the kidney[83], thus favours adhesion and retention of CaOx particles within the renal tubules.

Hence, in the present study, EG/AC in drinking water was employed to induce hyperoxaluria in rats. Urinary supersaturation in relation to stone forming constituents, mainly urinary oxalate is important in renal calculi formation[84], as urinary oxalic acid complexes with calcium and forms insoluble CaOx crystals[85]. Enhanced deposition and urinary excretion of calcium and oxalate in the preventive and curative control group animals indicate that administration of EG/AC induced hyperoxaluria. An increase in the kidney weight and enhanced urinary creatinine excretion in the control group animals also substantiated these results.

On administration of EPU, the dose-dependent reduction in calcium and oxalate deposition in the kidneys and their urinary excretion in control groups implies the potential of EPU. in preventing the formation and dissolving the preformed CaOx stones.

On treatment with the extract and standard cystone, the significant reduction in the elevated urinary creatinine, urea, uric acid, calcium, phosphorus, oxalate and magnesium in the treated groups reflects the improvement in hyperoxaluria induced renal impairment. Dissolution of calculi can be achieved by alteration in urinary pH[86]. If the pH is 5.0 or below, the stones likely to form are of uric acid type, if 5.0-6.5, calcium oxalate type and if above 7 indicates crystals of magnesium ammonium phosphate. In the present study, a decrease in the normal urine pH of 7.0-7.5 to 5.5-6.0 in the control groups, indicates hyperoxaluria induced CaOx stone formation. In the treated groups, EPU and

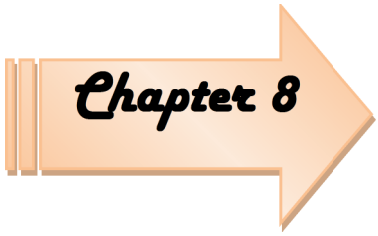
cystone 5ml/kg administration restored the pH to 6.5-7.5, supporting the decrease in the deposition and excretion of calcium and oxalate.

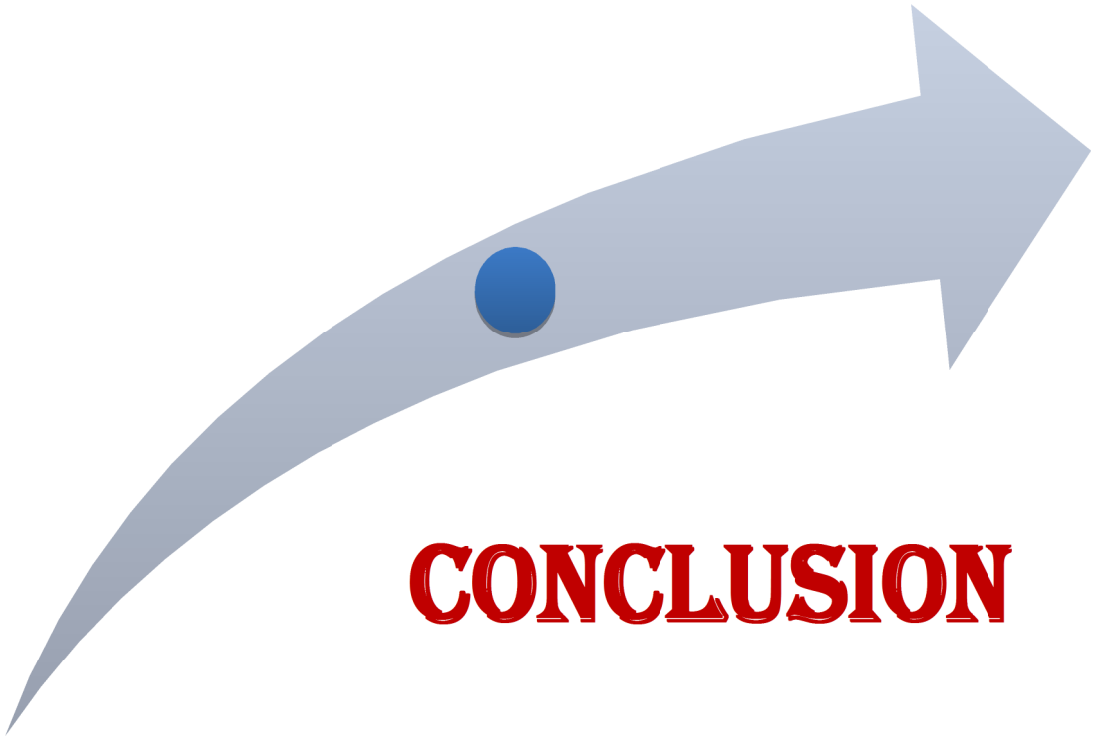
Mucoproteins have significant affinity for CaOx surface and promote the growth of crystals and cement them.[87] Flavonoids act by disintegrating the mucoprotiens, thereby prevent calcium and oxalate deposition and excretion[88]. In the present study also, preliminary phytochemical screening of EPU revealed the presence of flavonoids. Thus, in the EPU treated groups, flavonoids might have reduced calcium and oxalate deposition by pre-coating CaOx crystals and disintegrating the mucoproteins. The stone forming effects of EG are also ascribed to its hyperoxaluria induced oxidative damage. Oxalate has been reported to induce LPO and to cause renal tissue damage. As kidney is rich in polyunsaturated fatty acids, is susceptible to ROS attack. [89]. Excessive generation of ROS and/or a reduction in cellular antioxidant levels results in the development of OS.

MDA is one of the most common by products of ROS induced OS. In the present study, increased levels of MDA, diminished levels of GSH and catalase in the control groups indicate that EG/ AC administration promoted extensive generation of ROS. The resultant ROS may have consumed GSH and catalase excessively and impaired antioxidant protection. Thus, the unquenched ROS may have provoked cellular damage and resulted in enhanced OS, which might have further favoured the accumulation and retention of oxalate and subsequent deposition of CaOx. Studies show that treatment with antioxidants prevents CaOx deposition in the kidney and reduce Ox excretion. [90] Daily consumption of tea reduced the risk of kidney stone formation in women by 8%. [Moreover, low concentration of renal cellular glutathione favours LPO and subsequent retention of calcium and oxalate in the kidneys[91].

Health benefits of tea are due to its antioxidant properties of flavonoids [92] which act by quenching ROS and also by chelating metal ions like iron and copper. Lupeol and betulin were proposed to act by scavenging oxalate promoted free radicals and enhancing body antioxidant status, thus reducing oxalate induced renal peroxidative tissue damage. [93] In the present study, lowered levels of MDA and enhanced levels of antioxidant enzymes, GSH and catalase in the kidneys of the EPU treated animals indicate attenuation of hyperoxaluria induced LPO and oxidative damage. Flavonoids may have minimized ROS by free radical scavenging and prevented further generation, by metal chelating property,. Thus, the flavonoid principles of phyllanthus urinaria might have been responsible for the

inhibition of CaOx crystal aggregation and stone formation. The results support the use of Phyllanthus urinaria plant as an effective alternative in treating CaOx urolithiasis. Disintegration of the mucoproteins and pre-coating of CaOx crystals by antioxidant effect of flavonoid principles may be responsible for the possible antiurolithiatic activity of Phyllanthus urinaria. Further studies are necessary to find out the chemical components responsible for the antiurolithiatic activity of Phyllanthus urinaria L.



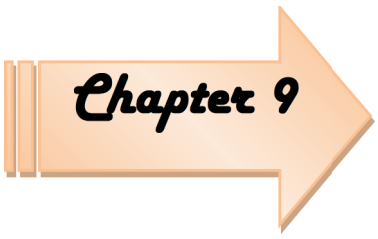


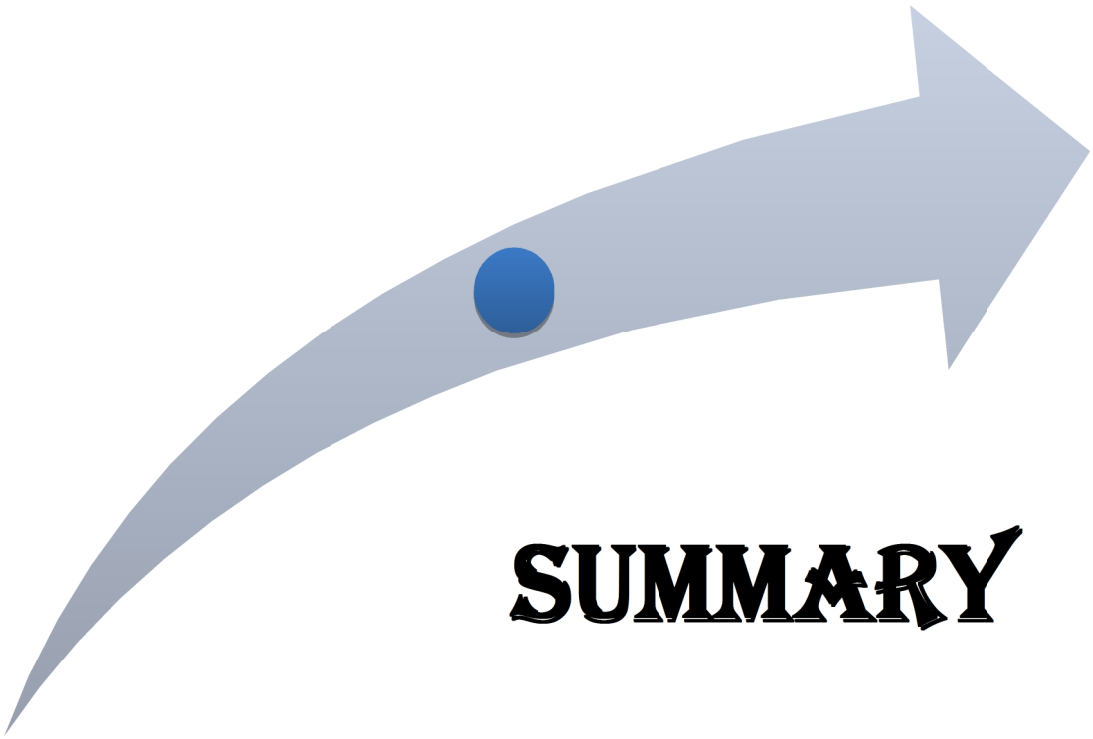
CONCLUSION

8. CONCLUSION

In conclusion, the presented data indicate that administration of the EPU plant to rats with ethylene glycol/Ammonium chloride induced lithiasis and prevented the formation of urinary stones, supporting folk information regarding antiurolithiatic activity of the plant. The mechanism underlying this effect is still unknown, but is apparently related to diuresis and lowering of urinary concentration of stone forming constituents. The protective effect against oxalate induced lipid peroxidation may be contributing to

the recovery of renal damage. These effects could conclude the antiurolithiatic property of Phyllanthus urinaria.





9. SUMMARY

In the present study, dried powder of Phyllanthus urinaria was subjected to extraction using 90% ethanol for the extraction. Some extract was reserved for preliminary phytochemical investigation and rest was utilized for pharmacological screening.

The preliminary phytochemical investigation showed the presence of alkaloids, carbohydrates, glycosides, saponins, and flavonoids.

The pharmacological screening included evaluation of antiurolithiatic activity using 0.75% ethylene glycol/ammonium chloride induced urolithiasis model in male Wistar albino rats. The kidney stone formation induced in rats, as a result of 10 days chronic administration of 0.75% and 2% ammonium chloride, was significantly inhibited by oral administration of EPU.

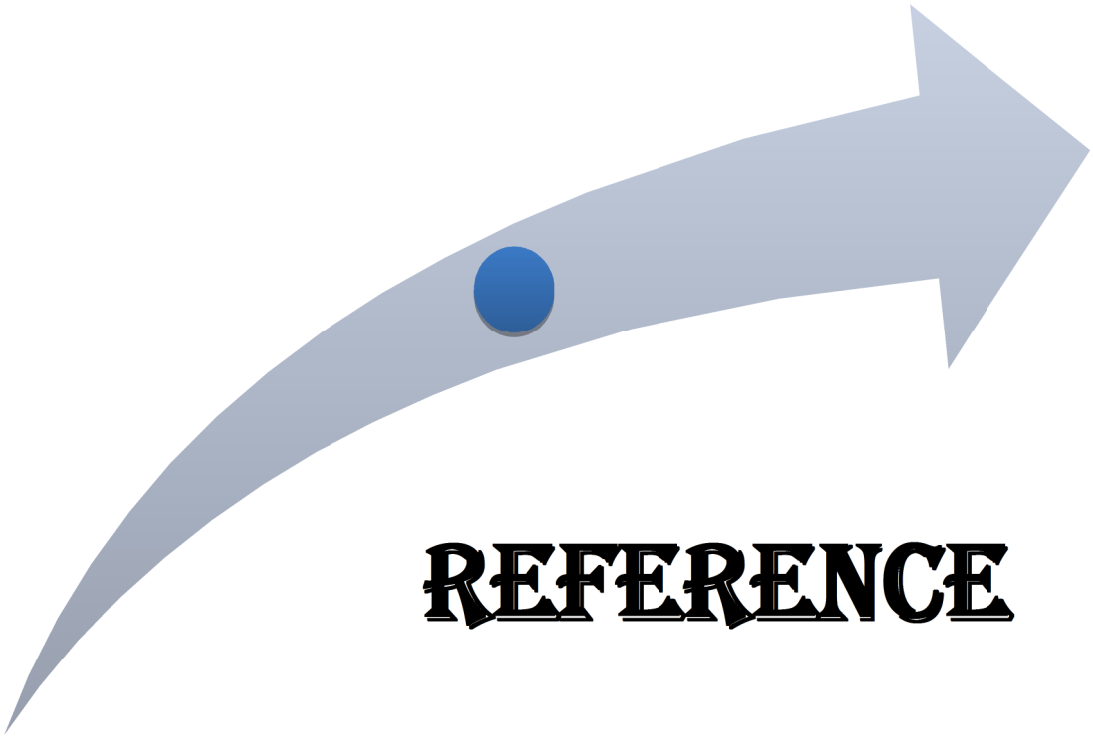
Administration of EPU and Cystone 5ml/kg caused significantly increased urine output and pH of the urine as compared to control (EG and AC) group.

Pretreatment with standard cystone 5ml/kg, EPU 200 and 400mg/kg causes significant reduction in serum creatinine, urea, uric acid, calcium, oxalate, phosphorus and magnesium concentration when compared to EG and AC alone treated group.

Pretreatment with standard cystone 5ml/kg, EPU 200 and 400mg/kg causes significant reduction in urinary creatinine, urea, uric acid, calcium, oxalate, phosphorus and magnesium concentration when compared to EG and AC alone treated group.

After treatment with standard cystone 5ml/kg and EPU 200 and 400mg/kg significant reduction in the kidney MDA levels was observed in the treated groups, when compared to their respective control groups.

Catalase and GSH levels of the kidney were significantly decreased in the control groups on EG/AC administration for 10 days, when compared to the normal group. On treatment with Standrad cystone and EPU 200 and 400mg/kg a significant rise in the renal catalase and GSH levels was observed in treated groups.



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AUTHENTICATION CERTIFICATE

I hereby certify that the following plant species for Pharmacognostical / Pharmaceutical / Pharmacological / Phytochemical / Biotechnological investigation research work is identified and their botanical name and family name is given.

Botanical name	Family
Phyllanthus urinaria L.	Phyllanthaceae

Authenticated by

Dr. K. Madhava Chetty

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APPROVAL CERTIFICATE

APPROVAL CERTIFICATE

This is to certify that the project title "***Evaluation of Antiurolithiatic activity of ethanolic extract of Phyllanthus urinaria against ethylene glycol induced urolithiasis in wistar albino rats***" has been approved (Approval No: 180/SICRA/IAEC) by the IAEC.

Chairman IAEC
(Dr.C.RAMESH)

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(*NOTE: Make sure that minutes of the meeting duly signed by all the IAEC members are maintained by the Office*)

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